

BEST AVAILABLE COPY

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
26 April 2001 (26.04.2001)

PCT

(10) International Publication Number
WO 01/29058 A1

- (51) International Patent Classification⁷: C07H 21/04,
C07K 14/435, 16/18, C12N 15/63, 15/67
- (21) International Application Number: PCT/US00/28470
- (22) International Filing Date: 13 October 2000 (13.10.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/159,776 15 October 1999 (15.10.1999) US
60/193,218 30 March 2000 (30.03.2000) US
- (71) Applicants: UNIVERSITY OF MASSACHUSETTS
[US/US]; One Beacon Street, 26th Floor, Boston, MA
02108 (US). CARNEGIE INSTITUTION OF WASH-
INGTON [US/US]; 115 West University Parkway,
Baltimore, MD 21210 (US).
- (72) Inventors: MELLO, Craig, C.; 19 Ryan Road, Shrews-
bury, MA 01545 (US). FIRE, Andrew; 2320 Bright Leaf
- Way, Baltimore, MD 21209 (US). TABARA, Hiroaki; 145
Orient Street, Worcester, MA 01545 (US). GRISHOK,
Alla; 18K Shrewsbury Green Drive, Shrewsbury, MA
01545 (US).
- (74) Agent: CREASON, Gary, L.; Fish & Richardson P.C.,
225 Franklin Street, Boston, MA 02110-2804 (US).
- (81) Designated States (national): AU, CA, JP, KR.
- (84) Designated States (regional): European patent (AT, BE,
CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC,
NL, PT, SE).
- Published:
— With international search report.
— Before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments.
- For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.



WO 01/29058 A1

(54) Title: RNA INTERFERENCE PATHWAY GENES AS TOOLS FOR TARGETED GENETIC INTERFERENCE

(57) Abstract: Genes involved in double-stranded RNA interference (RNAi pathway genes) are identified and used to investigate the RNAi pathway. The genes and their products are also useful for modulating RNAi pathway activity.

5

10

1

9:R599-R601; Sharp, 1999, *Genes & Dev.* 13:139-141) the mechanisms of action of specific components of the pathway are not known.

Attempts to overexpress a gene (e.g., a transgene) often lead only to transient expression of the gene. Furthermore, the even more undesirable effect of

5 "cosuppression" can occur in which a corresponding endogenous copy of the transgene becomes inactivated. In some cases, transgene silencing leads to problems with the commercial or therapeutic application of transgenic technology to alter the genetic makeup of a cell, organism, or human patient.

10 Summary of the Invention

The present invention relates to the discovery of RNA interference (RNAi) pathway genes which are involved in mediating double-stranded RNA-dependent gene silencing (genetic interference). RNAi requires a set of conserved cellular factors to suppress gene expression. These factors are the components of the RNAi pathway. The RNAi pathway mutations and genes described herein (e.g., *rde-1*, *rde-2*, *rde-3*, *rde-4*, *rde-5*, *mut-2*, and *mut-7*), and their protein products (e.g., RDE-1 and RDE-4) are useful tools for investigating the mechanisms involved in RNAi and developing methods of modulating the RNAi pathway. The sequences and methods described herein are useful for modulating the RNAi pathway and may be used in conjunction with other methods involving the use of genetic inhibition by dsRNA (e.g., see U.S.S.N. 09/215,257, filed December 18, 1998, incorporated herein by reference in its entirety).

RNAi pathway components (e.g., RDE-1, RDE-4) provide activities necessary for interference. These activities may be absent or not sufficiently activated in many cell types, including those of organisms such as humans in which genetic interference may have potential therapeutic value. Components of the RNAi pathway in *C. elegans* may be sufficient when provided through transgenesis or as direct RNA:protein complexes to activate or directly mediate genetic interference in heterologous cells that are deficient in RNAi.

Nucleic acid sequences encoding RNAi pathway components (e.g., RDE-1, RDE-4) are useful, e.g., for studying the regulation of the RNAi pathway. Such sequences can also be used to generate knockout strains of animals such as *C. elegans*.

The nucleic acids of the invention include nucleic acids that hybridize, e.g., under stringent hybridization conditions (as defined herein), to all or a portion of the nucleotide sequence of SEQ ID NO:1 (Figure 5A-C) or its complement; SEQ ID NO:2 (Figure 6A-D) or its complement, or SEQ ID NO:4 or its complement. The hybridizing portion of the hybridizing nucleic acids are preferably 20, 30, 50, or 70 bases long. Preferably, the hybridizing portion of the hybridizing nucleic acid is 80%, more preferably 95%, or even 98% or 100% identical to the sequence of a portion or all of a nucleic acid encoding an RDE-1 polypeptide or an RDE-4 polypeptide. Hybridizing nucleic acids of the type described above can be used as a cloning probe, a primer (e.g., a PCR primer), or a diagnostic probe. Preferred hybridizing nucleic acids encode a polypeptide having some or all of the biological activities possessed by a naturally-occurring RDE-1 polypeptide or an RDE-4 polypeptide e.g., as determined in the assays described below.

Hybridizing nucleic acids may encode a protein that is shorter or longer than the RDE-1 protein or RDE-4 protein described herein. Hybridizing nucleic acids may also encode proteins that are related to RDE-1 or RDE-4 (e.g., proteins encoded by genes that include a portion having a relatively high degree of identity to the *rde-1* gene or *rde-4* gene described herein).

The invention also features purified or isolated RDE-1 polypeptides and RDE-4 polypeptides. RDE-1 and RDE-4 polypeptides are useful for generating and testing antibodies that specifically bind to an RDE-1 or an RDE-4. Such antibodies can be used, e.g., for studying the RNAi pathway in *C. elegans* and other organisms. As used herein, both "protein" and "polypeptide" mean any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation). Thus, the term "RNAi pathway polypeptide" includes a full-length, naturally occurring RNAi pathway polypeptide such as RDE-1 protein or RDE-4 protein, as well as recombinantly or synthetically produced polypeptides that correspond to a full-length, naturally occurring RDE-1 protein, RDE-4 protein, or to particular domains or portions of a naturally occurring RNAi pathway protein.

RNAi pathway mutations and strains harboring those mutations (e.g., *rde-1*, *rde-2*, *rde-3*, *rde-4*, *rde-5*) are useful for studying the RNAi pathway, including identification of modulators of the RNAi pathway.

RNAi pathway components (e.g., those associated with *mut-7* and *rde-2*) can be used to desilence or prevent silencing of transgenes. To facilitate this function, such RNAi pathway components are inhibited using specific inhibitors of an RNAi pathway gene or its product.

5 In one embodiment, the invention includes an isolated nucleic acid molecule comprising a nucleotide sequence encoding an RDE-1 polypeptide. The nucleic acid molecule hybridizes under high stringency conditions to the nucleic acid sequence of Genbank Accession No. AF180730 (SEQ ID NO:2) or its complement, or the sequence of SEQ ID NO:1 or its complement. In one embodiment, the isolated nucleic acid can
10 complement an *rde-1* mutation. The invention also encompasses an isolated nucleic acid whose nucleotide sequence encodes the amino acid sequence of SEQ ID NO:3.

The invention also encompasses a substantially pure RDE-1 polypeptide encoded by the isolated nucleic acids described herein.

15 The invention features an antibody that specifically binds to an RDE-1 polypeptide.

The invention also includes a method of enhancing the expression of a transgene in a cell, the method comprising decreasing activity of the RNAi pathway. In one embodiment of this invention, *rde-2* expression or activity is decreased.

20 The invention also features an isolated nucleic acid molecule comprising a nucleotide sequence encoding an RDE-4 polypeptide, wherein the nucleic acid molecule hybridizes under high stringency conditions to the nucleic acid sequence of SEQ ID NO:4 or its complement. The invention also encompasses an isolated nucleic acid encoding an RDE-4 polypeptide, wherein the nucleic acid can complement an *rde-4* mutation. The invention also encompasses an isolated nucleic acid encoding an RDE-4
25 polypeptide, in which the nucleotide sequence encodes the amino acid sequence of SEQ ID NO:5.

The invention also features a substantially pure RDE-4 polypeptide encoded by the isolated nucleic acids described herein.

30 In another embodiment the invention features an antibody that specifically binds to an RDE-4 polypeptide.

The invention also features a method of preparing an RNAi agent, the method

includes incubating a dsRNA in the presence of an RDE-1 protein and an RDE-4 protein.

The invention also features a method of inhibiting the activity of a gene by introducing an RNAi agent into a cell, such that the dsRNA component of the RNAi agent is targeted to the gene. In another embodiment of the invention, the cell contains
5 an exogenous RNAi pathway sequence. The exogenous RNAi pathway sequence can be an RDE-1 polypeptide or an RDE-4 polypeptide. In still another embodiment, a dsRNA is introduced into a cell containing an exogenous RNAi pathway sequence such as nucleic acid sequence expressing an RDE-1 or RDE-4.

An RNAi pathway component is a protein or nucleic acid that is involved in
10 promoting dsRNA-mediated genetic interference. A nucleic acid component can be an RNA or DNA molecule. A mutation in a gene encoding an RNAi pathway component may decrease or increase RNAi pathway activity.

An RNAi pathway protein is a protein that is involved in promoting dsRNA mediated genetic interference.

15 A "substantially pure DNA" is a DNA that is not immediately contiguous with (i.e., covalently linked to) both of the coding sequences with which it is immediately contiguous (i.e., one at the 5' end and one at the 3' end) in the naturally-occurring genome of the organism from which the DNA of the invention is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an
20 autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR (polymerase chain reaction) or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequences.

25 By "inhibited RNAi pathway" is meant decreased inhibitory activity of a dsRNA which results in at least two-fold less inhibition by a dsRNA relative to its ability to cause inhibition in a wild type cell. Techniques for measuring RNAi pathway activity are described herein. The pathway can be inhibited by inhibiting a component of the pathway (e.g., RDE-1) or mutating the component so that its function is reduced.

30 A "substantially pure polypeptide" is a polypeptide, e.g., an RNAi pathway polypeptide or fragment thereof, that is at least 60%, by weight, free from the proteins

and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%; by weight, RNAi pathway polypeptide or fragment. A substantially pure RNAi pathway polypeptide or fragment thereof is obtained, for example, by extraction from a natural source; by expression of a recombinant nucleic acid encoding an RNAi pathway polypeptide or fragment thereof; or by chemically synthesizing the polypeptide or fragment. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

By "specifically binds" is meant a molecule that binds to a particular entity, e.g., an RNAi pathway polypeptide, but which does not substantially recognize or bind to other molecules in a sample, e.g., a biological sample, which includes the particular entity, e.g., RDE-1.

An RNAi agent is a dsRNA molecule that has been treated with those components of the RNAi pathway that are required to confer RNAi activity on the dsRNA. For example, treatment of a dsRNA under conditions that include RDE-1 and RDE-4 results in an RNAi agent. Injection of such an agent into an animal that is mutant for RDE-1 and RDE-4 will result in activation of the RNAi pathway with respect to a targeted gene. Typically, the dsRNA used to trigger the formation of the RNAi agent is selected to be an RNA corresponding to all or a portion of the nucleotide sequence of the targeted gene.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Brief Description of the Drawings

Figure 1A illustrates the genetic scheme used to identify *rde* mutants.

Figure 1B is an illustration summarizing data from the genetic mapping of *rde* and *mut* mutations. The vertical bars represent chromosomes; LGI, LGIII, and LGV.

5 Reference genetic markers are indicated at the right of each chromosome and the relative genetic positions of the *rde* and *mut* alleles are indicated at the left.

Figure 2A is a graphical representation of experiments investigating the sensitivity of *rde* and *mut* strains to RNAi by microinjection. The RNA species indicated above each graph was injected at high concentration (*pos-1*: 7mg/ml, *par-2*: 3mg/ml, *sqt-3*: 7mg/ml). The strains receiving injection are indicated at the left and the horizontal bar graphs reflect the percent of progeny that exhibited genetic interference. The *Unc* marker mutants used are also indicated. The percent embryonic lethality of F1 progeny is plotted as shaded bars and the fraction of affected progeny is indicated at the right of each graph.

Figure 2B is a graphical representation of experiments demonstrating that animals 15 homozygous for *rde* and *mut* alleles are resistant to RNAi targeting maternally expressed genes, *pos-1* and *par-2*. The percent embryonic lethality of F1 progeny is plotted as shaded bars and the fraction of affected progeny is indicated at the right of each graph.

Figure 3 is a schematic representation of homozygous *rde-1(ne219)* and *rde-4(ne299)* mutant mothers receiving injections of dsRNA targeting the body muscle 20 structural gene *unc-22*.

Figure 4A is a schematic representation of the physical map of the *rde-1* region. *C. elegans* YAC and cosmid DNA clones that were positive for rescue are indicated by an asterisk. A representation of the expanded interval showing a minimal, 25kb, rescuing interval defined by the overlap between cosmids T10A5 and C27H6 is shown beneath the 25 YAC and cosmid map. Predicted genes within this sequenced interval are illustrated above and below the hatch marked line. A single, rescuing, 4.5kb PCR fragment containing the KO8H10.7 predicted gene is shown enlarged. Exon and intron (box/line) boundaries are shown as well as the positions of *rde-1* point mutation in the predicted coding sequences.

30 Figure 4B is an illustration of the predicted sequence of RDE-1 and its alignment with four related proteins. The sequences are RDE-1 (*C. elegans*; Genbank Accession

No. AF180730), F48F7.1 (*C. elegans*; Genbank Accession No. Z69661), eIF2C (rabbit; Genbank Accession No. AF005355), ZWILLE (*Arabidopsis*; Genbank Accession No. AJ223508), and Sting (*Drosophila*; Genbank Accession No. AF145680). Identities with RDE-1 are shaded in black, and identities among the homologs are shaded in gray.

5 Figures 5A-5C are an illustration of the genomic sequence from cosmid KO8H10 (Genbank accession Z83113.1; SEQ ID NO:1) corresponding to the *rde-1* gene from the first nucleotide of 5' untranslated region to the polyadenylation site.

 Figures 6A-6D are an illustration of the cDNA sequence of *rde-1* (SEQ ID NO:2), including the first 20 nucleotides constituting the 5' untranslated sequence (5'UTR) and
10 the predicted amino acid sequence encoded by *rde-1* (RDE-1; SEQ ID NO:3). The nucleotide sequence is numbered starting with the first nucleotide of the translated region.

 Figure 7A is an illustration of the protocol for injection of a wild-type hermaphrodite with dsRNA.

15 Figure 7B is an illustration of a genetic scheme demonstrating extragenic inheritance of RNAi. The fraction shown represents the number of RNAi affected F2 hermaphrodites over the total number of cross progeny scored for each genotype class. Phenotypically uncoordinated (Unc).

 Figures 8A-8B are illustrations of a genetic scheme to determine if the wild-type
20 activities of *rde-1*, *rde-2*, *rde-4*, and *mut-7* are sufficient in the injected animal for interference among the F1 self progeny (A) illustrates crosses of heterozygous hermaphrodites; (B) illustrates crosses using homozygous F1 progeny from heterozygous mothers. The fraction shown represents the number of RNAi affected animals over the total number of cross progeny scored for each genotype class.

25 Figure 9A depicts experiments of a the genetic scheme to determine if the wild-type activities of *rde-1*, *rde-2*, *rde-4*, and *mut-7* are sufficient in the injected animal for interference among the F1 self progeny. The fraction shown represents the number of RNAi affected animals over the total number of cross progeny scored for each genotype class.

30 Figure 9B depicts experiments designed to determine the requirements for *rde-1*, *rde-2*, *rde-4*, and *mut-7* in F2 (Fig. 10A) and F1 (Fig. 10B) interference. The fraction

shown represents the number of RNAi affected animals over the total number of cross progeny scored for each genotype class.

Figures 10A-10B are a depiction of the cDNA sequence of a wild type *rde-4* nucleic acid sequence (SEQ ID NO:4) and the predicted RDE-4 amino acid sequence (SEQ ID NO:5) of *C. elegans*. “*” indicates ambiguous base assignment.

Figure 11 is a depiction of regions of homology between the predicted RDE-4 amino acid sequence, X1RBPA (SEQ ID NO:6), HsPKR (SEQ ID NO:7), and a consensus sequence (SEQ ID NO:8). A predicted secondary structure for RDE-4 is also shown illustrating predicted regions of α helix and β pleated sheet.

Figure 12 illustrates a scheme for rescue of an *rde-4*.

Detailed Description

Mutations have been discovered that identify genes involved in dsRNA-mediated genetic interference (RNAi). RNAi pathway genes encode products involved in genetic interference and are useful for mediating or enhancing genetic interference. These genes encode mediators of double-stranded RNA-mediated interference. The mediators can be nucleic acid or protein. RNAi pathway genes are also useful for mediating specific processes, e.g., a gene that mediates dsRNA uptake by cells may be useful for transporting other RNAs into cells or for facilitating entry of agents such as drugs into cells. The methods and examples described below illustrate the identification of RNAi pathway components, the uses of RNAi pathway components, mutants, genes and their products.

Identification of an RNAi-deficient mutants and an RNAi pathway gene, *rde-1*

RNAi pathway genes were identified using screens for *C. elegans* strains mutant for RNAi (Examples 2 and 3). The mutations were further characterized for germline and somatic effects, effects on transposon mobilization, X chromosome loss and transgene silencing, and target tissue activity (Examples 4 and 5).

The *rde-1* gene was identified using YACs (yeast artificial chromosomes) and cosmid to rescue *rde-1* mutants. Based on the identified sequence, a cDNA sequence

was identified in a *C. elegans* cDNA library and the complete cDNA sequence determined (Example 6).

Identification of RNAi Pathway Genes Homologous to rde-1, rde-2, rde-3, and rde-4

5 RNAi pathway genes from *C. elegans* (such as those described herein) and from other organisms (e.g. plant, mammalian, especially human) are useful for the elucidation of the biochemical pathways involved in genetic interference and for developing the uses of RNAi pathway genes described herein.

Several approaches can be used to isolate RNAi pathway genes including two-
10 hybrid screens, complementation of *C. elegans* mutants by expression libraries of cloned heterologous (e.g., plant, mammalian, human) cDNAs, polymerase chain reactions (PCR) primed with degenerate oligonucleotides, low stringency hybridization screens of heterologous cDNA or genomic libraries with a *C. elegans* RNAi pathway gene, and database screens for sequences homologous to an RNAi pathway gene. Hybridization is
15 performed under stringent conditions. Alternatively, a labeled fragment can be used to screen a genomic library derived from the organism of interest, again, using appropriately stringent conditions. Such stringent conditions are well known, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived.

20 Nucleic acid duplex or hybrid stability is expressed as the melting temperature or T_m , which is the temperature at which a probe dissociates from a target DNA. This melting temperature is used to define the required stringency conditions. If sequences are to be identified that are related and substantially identical to the probe, rather than identical, then it is useful to first establish the lowest temperature at which only
25 homologous hybridization occurs with a particular SSC or SSPE concentration. Then assume that 1% mismatching results in 1°C decrease in the T_m and reduce the temperature of the final wash accordingly (for example, if sequences with $\geq 95\%$ identity with the probe are sought, decrease the final wash temperature by 5°C). Note that this assumption is very approximate, and the actual change in T_m can be between 0.5° and 1.5°C per 1%
30 mismatch.

As used herein, high stringency conditions include hybridizing at 68°C in 5x SSC/5x Denhardt solution/1.0% SDS, or in 0.5 M NaHPO₄ (pH 7.2)/1 mM EDTA/7% SDS, or in 50% formamide/0.25 M NaHPO₄ (pH 7.2)/0.25 M NaCl/1 mM EDTA/7% SDS; and washing in 0.2x SSC/0.1% SDS at room temperature or at 42°C, or in 0.1x SSC/0.1% SDS at 68°C, or in 40 mM NaHPO₄ (pH 7.2)/1 mM EDTA/5% SDS at 50°C, or in 40 mM NaHPO₄ (pH 7.2) 1 mM EDTA/1% SDS at 50°C. Moderately stringent conditions include washing in 3x SSC at 42°C. The parameters of salt concentration and temperature can be varied to achieve the desired level of identity between the probe and the target nucleic acid.

For guidance regarding such conditions see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Springs Harbor Press, N.Y.; and Ausubel et al. (eds.), 1995, Current Protocols in Molecular Biology, (John Wiley & Sons, N.Y.) at Unit 2.10.

Methods of screening for and identifying homologs of *C. elegans* RNAi genes (e.g., *rde-1*) are known in the art. For example, complementation of mutants, described in the Examples can be performed using nucleic acid sequences from organisms other than *C. elegans*. Methods of inhibiting expression of a target gene in a cell using dsRNA are known in the art and are exemplified in U.S.S.N. 09/215,257, filed December 18, 1998, which is incorporated herein by reference in its entirety.

Another method of screening is to use an identified RNAi pathway gene sequence to screen a cDNA or genomic library using low stringency hybridizations. Such methods are known in the art.

PCR with degenerate oligonucleotides is another method of identifying homologs of RNAi pathway genes (e.g., human *rde-1*). Homologs of an RNAi pathway gene identified in other species are compared to identify specific regions with a high degree of homology (as in the sequence comparison shown in Figure 4). These regions of high homology are selected for designing PCR primers that maximize possible base-pairing with heterologous genes. Construction of such primers involves the use of oligonucleotide mixtures that account for degeneracy in the genetic code, i.e., allow for the possible base changes in an RNAi pathway gene that does not affect the amino acid sequence of the RNAi pathway protein. Such primers may be used to amplify and clone

possible RNAi pathway gene fragments from DNA isolated from another organism (e.g., mouse or human). The latter are sequenced and those encoding protein fragments with high degrees of homology to fragments of the RNAi pathway protein are used as nucleic acid probes in subsequent screens of genomic DNA and cDNA libraries (e.g., mouse or human). Full-length genes and cDNAs having substantial homology to the previously identified RNAi pathway gene are identified in these screens.

To produce an RNAi pathway gene product (e.g., RDE-1) a sequence encoding the gene is placed in an expression vector and the gene expressed in an appropriate cell type. The gene product is isolated from such cell lines using methods known to those in the art, and used in the assays and procedures described herein. The gene product can be a complete RNAi pathway protein (e.g., RDE-1) or a fragment of such a protein.

Methods of Expressing RNAi Pathway Proteins

Full-length polypeptides and polypeptides corresponding to one or more domains of a full-length RNAi pathway protein, e.g., the RNA-binding domain of RDE-4, are also within the scope of the invention. Also within the invention are fusion proteins in which a portion (e.g., one or more domains) of an RDE-1 or RDE-4) is fused to an unrelated protein or polypeptide (i.e., a fusion partner) to create a fusion protein. The fusion partner can be a moiety selected to facilitate purification, detection, or solubilization, or to provide some other function. Fusion proteins are generally produced by expressing a hybrid gene in which a nucleotide sequence encoding all or a portion of of an RNAi pathway protein is joined in-frame to a nucleotide sequence encoding the fusion partner. Fusion partners include, but are not limited to, the constant region of an immunoglobulin (IgFc). A fusion protein in which an RNAi pathway polypeptide is fused to IgFc can be more stable and have a longer half-life in the body than the polypeptide on its own.

In general, RNAi pathway proteins (e.g., RDE-1, RDE-4) according to the invention can be produced by transformation (transfection, transduction, or infection) of a host cell with all or part of an RNAi pathway protein-encoding DNA fragment (e.g., one of the cDNAs described herein) in a suitable expression vehicle. Suitable expression vehicles include: plasmids, viral particles, and phage. For insect cells, baculovirus expression vectors are suitable. The entire expression vehicle, or a part thereof, can be

integrated into the host cell genome. In some circumstances, it is desirable to employ an inducible expression vector, e.g., the LACSWITCH™ Inducible Expression System (Stratagene; LaJolla, CA).

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems can be used to provide the recombinant protein. The precise host cell used is not critical to the invention. The RNAi pathway protein can be produced in a prokaryotic host (e.g., *E. coli* or *B. subtilis*) or in a eukaryotic host (e.g., *Saccharomyces* or *Pichia*; mammalian cells, e.g., COS, NIH 3T3 CHO, BHK, 293, or HeLa cells; or insect cells).

Proteins and polypeptides can also be produced in plant cells. For plant cells viral expression vectors (e.g., cauliflower mosaic virus and tobacco mosaic virus) and plasmid expression vectors (e.g., Ti plasmid) are suitable. Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, MD; also, *see*, e.g., Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1994). The methods of transformation or transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al., *supra*; expression vehicles may be chosen from those provided, e.g., in *Cloning Vectors: A Laboratory Manual* (P.H. Pouwels et al., 1985, Supp. 1987).

The host cells harboring the expression vehicle can be cultured in conventional nutrient media adapted as need for activation of a chosen gene, repression of a chosen gene, selection of transformants, or amplification of a chosen gene.

One preferred expression system is the mouse 3T3 fibroblast host cell transfected with a pMAMneo expression vector (Clontech, Palo Alto, CA). pMAMneo provides an RSV-LTR enhancer linked to a dexamethasone-inducible MMTV-LTR promoter, an SV40 origin of replication which allows replication in mammalian systems, a selectable neomycin gene, and SV40 splicing and polyadenylation sites. DNA encoding an RNAi pathway protein would be inserted into the pMAMneo vector in an orientation designed to allow expression. The recombinant RNAi pathway protein would be isolated as described herein. Other preferable host cells that can be used in conjunction with the

pMAMneo expression vehicle include COS cells and CHO cells (ATCC Accession Nos. CRL 1650 and CCL 61, respectively).

RNAi pathway polypeptides can be produced as fusion proteins. For example, the expression vector pUR278 (Ruther et al., *EMBO-J.* 2:1791, 1983), can be used to create
5 lacZ fusion proteins. The pGEX vectors can be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can be easily purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target
10 gene product can be released from the GST moiety.

In an insect cell expression system, Autographa californica nuclear polyhydrosis virus (AcNPV), which grows in Spodoptera frugiperda cells, is used as a vector to express foreign genes. An RNAi pathway protein coding sequence can be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and
15 placed under control of an AcNPV promoter, e.g., the polyhedrin promoter. Successful insertion of a gene encoding an RNAi pathway polypeptide or protein will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat encoded by the polyhedrin gene). These recombinant viruses are then used to infect spodoptera frugiperda cells in which the
20 inserted gene is expressed (see, e.g., Smith et al., *J. Virol.* 46:584, 1983; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems can be utilized. When an adenovirus is used as an expression vector, the RNAi pathway protein nucleic acid sequence can be ligated to an adenovirus transcription/ translation control
25 complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene can then be inserted into the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion into a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing an RNAi pathway gene product in infected hosts (see, e.g., Logan, *Proc. Natl. Acad. Sci. USA* 81:3655, 1984).

30 Specific initiation signals may be required for efficient translation of inserted nucleic acid sequences. These signals include the ATG initiation codon and adjacent

sequences. In cases where an entire native RNAi pathway protein gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. In other cases, exogenous translational control signals, including, perhaps, the ATG initiation
5 codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators
10 (Bittner et al., *Methods in Enzymol.* 153:516, 1987).

RNAi pathway polypeptides can be expressed directly or as a fusion with a heterologous polypeptide, such as a signal sequence or other polypeptide having a specific cleavage site at the N-and/or C-terminus of the mature protein or polypeptide. Included within the scope of this invention are RNAi pathway polypeptides with a
15 heterologous signal sequence. The heterologous signal sequence selected should be one that is recognized and processed, i.e., cleaved by a signal peptidase, by the host cell. For prokaryotic host cells a prokaryotic signal sequence is selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion a yeast invertase, alpha factor, or acid phosphatase leaders may be
20 selected. In mammalian cells, it is generally desirable to select a mammalian signal sequences.

A host cell may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in a specific, desired fashion. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein
25 products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper
30 processing of the primary transcript, glycosylation, and phosphorylation of the gene product can be used. Such mammalian host cells include, but are not limited to, CHO,

VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, choroid plexus cell lines.

Alternatively, an RNAi pathway protein can be produced by a stably-transfected mammalian cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public, see, e.g., Pouwels et al. (supra); methods for constructing such cell lines are also publicly available, e.g., in Ausubel et al. (supra). In one example, cDNA encoding an RNAi pathway protein (e.g., RDE-1 or RDE-4) is cloned into an expression vector that includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the RNAi pathway protein-encoding gene into the host cell chromosome is selected for by including 0.01-300 μ M methotrexate in the cell culture medium (as described in Ausubel et al., supra). This dominant selection can be accomplished in most cell types.

Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (supra); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for this purpose include pCVSEII-DHFR and pAdd26SV(A) (described in Ausubel et al., supra). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR⁻ cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

A number of other selection systems can be used, including but not limited to the herpes simplex virus thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase, and adenine phosphoribosyltransferase genes can be employed in *tk*, *hgprt*, or *aprt* cells, respectively. In addition, *gpt*, which confers resistance to mycophenolic acid (Mulligan et al., *Proc. Natl. Acad. Sci. USA*, 78:2072, 1981); *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., *J. Mol. Biol.*, 150:1, 1981); and *hygro*, which confers resistance to hygromycin (Santerre et al., *Gene*, 30:147, 1981), can be used.

Alternatively, any fusion protein can be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described in

Janknecht et al., *Proc. Natl. Acad. Sci. USA*, **88**:8972 (1981), allows for the ready purification of non-denatured fusion proteins expressed in human cell lines. In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni²⁺ nitriloacetic acid-agarose columns, and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

Alternatively, an RNAi pathway protein or a portion thereof, can be fused to an immunoglobulin Fc domain. Such a fusion protein can be readily purified using a protein A column.

Antibodies that Recognize RNAi Pathway Proteins

Techniques for generating both monoclonal and polyclonal antibodies specific for a particular protein are well known. The invention also includes humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, and molecules produced using a Fab expression library.

Antibodies can be raised against a short peptide epitope of an RNAi pathway gene (e.g., rde-1), an epitope linked to a known immunogen to enhance immunogenicity, a long fragment of an RNAi pathway gene, or the intact protein. Such antibodies are useful for e.g., localizing RNAi pathway polypeptides in tissue sections or fractionated cell preparations, determining whether an RNAi pathway gene is expressed (e.g., after transfection with an RNAi pathway gene), and evaluating the expression of an RNAi pathway gene in disorders (e.g., genetic conditions) where the RNAi pathway may be affected.

An isolated RNAi pathway protein (e.g., RDE-1), or a portion or fragment thereof, can be used as an immunogen to generate antibodies that bind to an RNAi pathway protein using standard techniques for polyclonal and monoclonal antibody preparation. The RNAi pathway immunogen can also be a mutant RNAi pathway protein or a fragment of a mutant RNAi pathway protein. A full-length RNAi pathway protein can be used or, alternatively, antigenic peptide fragments of RNAi pathway protein can be used as immunogens. The antigenic peptide of an RNAi pathway protein comprises at

least 8 (preferably 10, 15, 20, or 30) amino acid residues. In the case of RDE-1, these residues are drawn from the amino acid sequence shown in SEQ ID NO:3 and encompass an epitope such that an antibody raised against the peptide forms a specific immune complex with RDE-1. Preferred epitopes encompassed by the antigenic peptide are regions of the protein that are located on the surface of the protein, e.g., hydrophilic regions.

An RNAi pathway protein immunogen typically is used to prepare antibodies by immunizing a suitable subject (e.g., rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, recombinantly expressed RNAi pathway protein or a chemically synthesized RNAi polypeptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic RNAi pathway protein preparation induces a polyclonal anti-RNAi pathway protein antibody response.

Polyclonal antibodies that recognize an RNAi pathway protein ("RNAi pathway antibodies") can be prepared as described above by immunizing a suitable subject with an RNAi pathway protein immunogen. The RNAi pathway antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme-linked immunosorbent assay (ELISA) using immobilized RNAi pathway protein from which the immunogen was derived. If desired, the antibody molecules directed against the RNAi pathway protein can be isolated from the mammal (e.g., from the blood) and further purified by well-known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, e.g., when the RNAi pathway antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497, the human B cell hybridoma technique (Kozbor et al. (1983) *Immunol. Today* 4:72), the EBV-hybridoma technique (Cole et al. (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing hybridomas is well known (see generally *Current Protocols in Immunology* (1994) Coligan et al. (eds.) John Wiley & Sons, Inc., New York, NY). Briefly, an

immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with an RNAi pathway immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds to the RNAi pathway protein.

5 Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating a monoclonal antibody against an RNAi pathway protein (see, e.g., *Current Protocols in Immunology*, supra; Galfre et al., 1977, *Nature* 266:55052; R.H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York, 1980; and Lerner, 1981, *Yale J. Biol. Med.*, 54:387-402. Moreover, one in the art will appreciate that there are many variations of such methods which also would be useful. Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind to the RNAi pathway protein, e.g., using a standard ELISA assay.

15 Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal RNAi pathway antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with an RNAi pathway protein to thereby isolate immunoglobulin library members that bind to the RNAi pathway protein. Kits for generating and screening phage display libraries are commercially available (e.g., the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP™ Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, U.S. Patent No. 5,223,409; PCT Publication No. WO 92/18619; 20 PCT Publication No. WO 91/17271; PCT Publication No. WO 92/20791; PCT Publication No. WO 92/15679; PCT Publication No. WO 93/01288; PCT Publication No. WO 92/01047; PCT Publication No. WO 92/09690; PCT Publication No. WO 90/02809; Fuchs et al., 1991, *Bio/Technology* 9:1370-1372; Hay et al., 1992, *Hum. Antibod. Hybridomas* 3:81-85; Huse et al., 1989, *Science* 246:1275-1281; Griffiths et al., 1993, 25 *EMBO J.* 12:725-734.

Techniques developed for the production of "chimeric antibodies" (Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851, 1984; Neuberger et al., *Nature*, 312:604, 1984; Takeda et al., *Nature*, 314:452, 1984) can be used to splice the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; and U.S. Patents 4,946,778 and 4,704,692) can be adapted to produce single chain antibodies against an RNAi pathway protein or polypeptide. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments that recognize and bind to specific epitopes can be generated by known techniques. For example, such fragments can include but are not limited to F(ab')₂ fragments, which can be produced by pepsin digestion of the antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of F(ab')₂ fragments. Alternatively, Fab expression libraries can be constructed (Huse et al., *Science*, 246:1275, 1989) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Identification of RNAi Pathway Components

RNAi pathway components can be identified in *C. elegans* and other animals (e.g., a mammal) using the methods described in the Examples below. Pathway components can also be identified using methods known in the art and the information provided herein. Such components include those involved in protein:protein and protein:RNA interactions. Specifically, RDE-1 can be used to identify additional proteins and RNA molecules that bind to the RDE-1 protein and so facilitate genetic interference.

The RNAi pathway mutant strains described herein (e.g., *rde-1*, *rde-2*, *rde-3*, *rde-4*, and *rde-5*; also *mut-2* and *mut-7*) can be used in genetic screens to identify additional

RNAi pathway components. For example, a strain deficient for rde-1 activity can be mutagenized and screened for the recovery of genetic interference. This type of screen can identify allele-specific suppressors in other genes or second site mutations within the rde-1 gene that restore its activity. The resulting strains may define new genes that
5 activate RNAi to overcome or bypass the rde-1 defect. The mutations identified by these methods can be used to identify their corresponding gene sequences.

Two-hybrid screens can also be used to identify proteins that bind to RNAi pathway proteins such as RDE-1. Genes encoding proteins that interact with RDE-1 or human homologs of the *C. elegans* RDE-1, are identified using the two-hybrid method
10 (Fields and Song, 1989, *Nature* 340:245-246; Chien et al., 1991, *Proc. Natl. Acad. Sci. USA* 88:9578-9582; Fields and Sternglanz, 1994, *Trends Genet.* 10:286-292; Bartel and Fields, 1995, *Methods Enzymol.* 254:241-263). DNA encoding the RDE-1 protein is cloned and expressed from plasmids harboring GAL4 or lexA DNA-binding domains and co-transformed into cells harboring lacZ and HIS3 reporter constructs along with libraries
15 of cDNAs that have been cloned into plasmids harboring the GAL4 activation domain. Libraries used for such co-transformation include those made from *C. elegans* or a vertebrate embryonic cell.

Mechanisms of Action of RNAi Pathway Components

20 Specific cellular functions associated with the RNAi pathway include the specific targeting of a nucleic acid by a dsRNA, uptake of dsRNA, transport of dsRNA, amplification of the dsRNA signal, and genetic interference. The mechanism of interference may involve translation inhibition, or interference with RNA processing. In addition, direct effects on the corresponding gene may contribute to interference. These
25 mechanisms can be identified investigated using the methods described herein and methods known in the art.

Methods of Screening for Molecules that Inhibit the RNAi Pathway

The following assays are designed to identify compounds that are effective
30 inhibitors of the RNAi pathway. Such inhibitors may act by, but are not limited to, binding to an RDE-1 polypeptide (e.g., from *C. elegans*, mouse, or human), binding to

intracellular proteins that bind to an RNAi pathway component, compounds that interfere with the interaction between RNAi pathway components including between an RNAi pathway component and a dsRNA, and compounds that modulate the activity or expression of an RNAi pathway gene such as *rde-1*. An inhibitor of the RNAi pathway
5 can also be used to promote expression of a transgene.

Assays can also be used to identify molecules that bind to RNAi pathway gene regulatory sequences (e.g., promoter sequences), thus modulating gene expression. See, e.g., Platt, 1994, *J. Biol. Chem.* 269:28558-28562, incorporated herein by reference in its entirety.

10 The compounds which may be screened by the methods described herein include, but are not limited to, peptides and other organic compounds (e.g., peptidomimetics) that bind to an RNAi pathway protein (e.g., that bind to an RDE-1), or inhibit its activity in any way.

Such compounds may include, but are not limited to, peptides; for example,
15 soluble peptides, including but not limited to members of random peptide libraries; (see, e.g., Lam et al., 1991, *Nature* 354:82-94; Houghten et al., 1991, *Nature* 354:84-86), and combinatorial chemistry-derived molecular libraries made of D-and/or L-amino acids, phosphopeptides (including, but not limited to, members of random or partially degenerate, directed phosphopeptide libraries; see e.g., Songyang et al., 1993, *Cell*
20 72:767-778), and small organic or inorganic molecules.

Organic molecules are screened to identify candidate molecules that affect expression of an RNAi pathway gene (e.g., *rde-1*), e.g., by interacting with the regulatory region or transcription factors of a gene. Compounds are also screened to identify those that affect the activity of such proteins, (e.g., by inhibiting *rde-1* activity) or the activity
25 of a molecule involved in the regulation of, for example, *rde-1*.

Computer modeling or searching technologies are used to identify compounds, or identify modifications of compounds that modulate the expression or activity of an RNAi pathway protein. For example, compounds likely to interact with the active site of a protein (e.g., RDE-1) are identified. The active site of an RNAi pathway protein can be
30 identified using methods known in the art including, for example, analysis of the amino acid sequence of a molecule, from a study of complexes of an RNAi pathway, with its

native ligand (e.g., a dsRNA). Chemical or X-ray crystallographic methods can be used to identify the active site of an RNAi pathway protein by the location of a bound ligand such as a dsRNA.

The three-dimensional structure of the active site is determined. This can be done using known methods, including X-ray crystallography which may be used to determine a complete molecular structure. Solid or liquid phase NMR can be used to determine certain intra-molecular distances. Other methods of structural analysis can be used to determine partial or complete geometrical structures. Geometric structure can be determined with an RNAi pathway protein bound to a natural or artificial ligand which may provide a more accurate active site structure determination.

Computer-based numerical modeling can also be used to predict protein structure (especially of the active site), or be used to complete an incomplete or insufficiently accurate structure. Modeling methods that may be used are, for example, parameterized models specific to particular biopolymers such as proteins or nucleic acids, molecular dynamics models based on computing molecular motions, statistical mechanics models based on thermal ensembles, or combined models. For most types of models, standard molecular force fields, representing the forces between constituent atoms and groups are necessary, and can be selected for the model from among the force fields known in physical chemistry. Information on incomplete or less accurate structures determined as above can be incorporated as constraints on the structures computed by these modeling methods.

Having determined the structure of the active site of an RNAi pathway protein (e.g., RDE-1), either experimentally, by modeling, or by a combination of methods, candidate modulating compounds can be identified by searching databases containing compounds along with information on their molecular structure. The compounds identified in such a search are those that have structures that match the active site structure, fit into the active site, or interact with groups defining the active site. The compounds identified by the search are potential RNAi pathway modulating compounds.

These methods may also be used to identify improved modulating compounds from an already known modulating compound or ligand. The structure of the known compound is modified and effects are determined using experimental and computer

modeling methods as described above. The altered structure may be compared to the active site structure of an RNAi pathway protein (e.g., an RDE-1) to determine or predict how a particular modification to the ligand or modulating compound will affect its interaction with that protein. Systematic variations in composition, such as by varying side groups, can be evaluated to obtain modified modulating compounds or ligands of preferred specificity or activity.

Other experimental and computer modeling methods useful to identify modulating compounds based on identification of the active sites of an RNAi pathway protein and related transduction and transcription factors will be apparent to those of skill in the art.

Examples of molecular modeling systems are the QUANTA programs, e.g., CHARMM, MCSS/HOOK, and X-LIGAND, (Molecular Simulations, Inc., San Diego, CA). QUANTA analyzes the construction, graphic modeling, and analysis of molecular structure. CHARMM analyzes energy minimization and molecular dynamics functions. MCSS/HOOK characterizes the ability of an active site to bind a ligand using energetics calculated via CHARMM. X-LIGAND fits ligand molecules to electron density of protein-ligand complexes. It also allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

Articles reviewing computer modeling of compounds interacting with specific protein can provide additional guidance. For example, see Rotivinen et al., 1988, *Acta Pharmaceutical Fennica* 97:159-166; Ripka, *New Scientist* June 16, 1988 pp.54-57; McKinaly and Rossmann, 1989, *Ann. Rev. Pharmacol. Toxicol.* 29:111-122; Perry and Davies. *OSAR Quantitative Structure -Activity Relationships in Drug Design* pp. 189-193 (Alan R. Liss, Inc., 1989); Lewis and Dean, 1989, *Proc. R. Soc. Lond.* 236:125-140, 141-152; and, regarding a model receptor for nucleic acid components, Askew et al., *Am. J. Chem. Soc.* 111:1082-1090. Computer programs designed to screen and depict chemicals are available from companies such as MSI (*supra*), Allelix, Inc. (Mississauga, Ontario, Canada), and Hypercube, Inc. (Gainesville, FL).

These applications are largely designed for drugs specific to particular proteins; however, they can be adapted to the design of drugs specific to identified regions of DNA or RNA. Chemical libraries that can be used in the protocols described herein include

those available, e.g., from ArQule, Inc. (Medford, MA) and Oncogene Science, Inc. (Uniondale, NY).

In addition to designing and generating compounds that alter binding, as described above, libraries of known compounds, including natural products, synthetic chemicals, and biologically active materials including peptides, can be screened for compounds that are inhibitors or activators of the RNAi pathway components identified herein.

Compounds identified by methods described above can be used, for example, for elaborating the biological function of RNAi pathway gene products (e.g., an RDE-1), and to treat genetic disorders involving an RNAi pathway protein. Assays for testing the effectiveness of compounds such as those described herein are further described below.

In vitro Screening Assays for Compounds that Bind to RNAi Pathway Proteins and Genes

In vitro systems can be used to identify compounds that interact with (e.g., bind to) RNAi pathway proteins or genes encoding those proteins (e.g., rde-1 and its protein product). Such compounds are useful, for example, for modulating the activity of these entities, elaborating their biochemistry, treating disorders in which a decrease or increase in dsRNA mediated genetic interference is desired. Such compounds may also be useful to treat diseases in animals, especially humans, involving nematodes, e.g., trichinosis, trichuriasis, and toxocariasis. Compounds such as those described herein may also be useful to treat plant diseases caused by nematodes. These compounds can be used in screens for compounds that disrupt normal function, or may themselves disrupt normal function.

Assays to identify compounds that bind to RNAi pathway proteins involve preparation of a reaction mixture of the protein and the test compound under conditions sufficient to allow the two components to interact and bind, thus forming a complex which can be removed and/or detected.

Screening assays can be performed using a number of methods. For example, an RNAi pathway protein from an organism (e.g., RDE-1), peptide, or fusion protein can be immobilized onto a solid phase, reacted with the test compound, and complexes detected

by direct or indirect labeling of the test compound. Alternatively, the test compound can be immobilized, reacted with the RNAi pathway molecule, and the complexes detected. Microtiter plates may be used as the solid phase and the immobilized component anchored by covalent or noncovalent interactions. Non-covalent attachment may be achieved by coating the solid phase with a solution containing the molecule and drying. Alternatively, an antibody, for example, one specific for an RNAi pathway protein such as RDE-1 is used to anchor the molecule to the solid surface. Such surfaces may be prepared in advance of use, and stored.

In these screening assays, the non-immobilized component is added to the coated surface containing the immobilized component under conditions sufficient to permit interaction between the two components. The unreacted components are then removed (e.g., by washing) under conditions such that any complexes formed will remain immobilized on the solid phase. The detection of the complexes may be accomplished by a number of methods known to those in the art. For example, the nonimmobilized component of the assay may be prelabeled with a radioactive or enzymatic entity and detected using appropriate means. If the non-immobilized entity was not prelabeled, an indirect method is used. For example, if the non-immobilized entity is an RDE-1, an antibody against the RDE-1 is used to detect the bound molecule, and a secondary, labeled antibody used to detect the entire complex.

Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected (e.g., using an immobilized antibody specific for an RNAi pathway protein).

Cell-based assays can be used to identify compounds that interact with RNAi pathway proteins. Cell lines that naturally express such proteins or have been genetically engineered to express such proteins (e.g., by transfection or transduction of an rde-1 DNA) can be used. For example, test compounds can be administered to cell cultures and the amount of mRNA derived from an RNAi pathway gene analyzed, e.g., by Northern analysis. An increase in the amount of RNA transcribed from such a gene compared to control cultures that did not contain the test compound indicates that the test compound is an inhibitor of the RNAi pathway. Similarly, the amount of a polypeptide encoded by an RNAi pathway gene, or the activity of such a polypeptide, can be analyzed

in the presence and absence of a test compound. An increase in the amount or activity of the polypeptide indicates that the test compound is an inhibitor of the RNAi pathway.

Ectopic Expression of an RNAi Pathway Gene

5 Ectopic expression (i.e., expression of an RNAi pathway gene in a cell where it is not normally expressed or at a time when it is not normally expressed) of a mutant RNAi pathway gene (i.e., an RNAi pathway gene that suppresses genetic interference) can be used to block or reduce endogenous interference in a host organism. This is useful, e.g., for enhancing transgene expression in those cases where the RNAi pathway is interfering
10 with expression of a transgene. Another method of accomplishing this is to knockout or down regulate an RNAi pathway gene using methods known in the art. These methods are useful in both plants and animals (e.g., in an invertebrate such as a nematode, a mouse, or a human).

 Ectopic expression of an RNAi pathway gene, e.g., *rde-1* or *rde-4* can also be
15 used to activate the RNAi pathway. In some cases, targeting can be used to activate the pathway in specific cell types, e.g., tumor cells. For example, a non-viral RNAi pathway gene construct can be targeted *in vivo* to specific tissues or organs, e.g., the liver or muscle, in patients. Examples of delivery systems for targeting such constructs include receptor mediated endocytosis, liposome encapsulation (described below), or direct
20 insertion of non-viral expression vectors.

 An example of one such method is liposome encapsulation of nucleic acid. Successful *in vivo* gene transfer has been achieved with the injection of DNA, e.g., as a linear construct or a circular plasmid, encapsulated in liposomes (Ledley, Human Gene Therapy 6:1129-1144 (1995) and Farhood, et al., Ann. NY Acad. Sci. 716:23-35 (1994)).
25 A number of cationic liposome amphiphiles are being developed (Ledley, Human Gene Therapy 6:1129-1144 (1995); Farhood, et al., Ann. NY Acad. Sci., 716:23-35 (1994) that can be used for this purpose.

 Targeted gene transfer has been shown to occur using such methods. For example, intratracheal administration of cationic lipid-DNA complexes was shown to
30 effect gene transfer and expression in the epithelial cells lining the bronchus (Brigham, et al., Am. J. Respir. Cell Mol. Biol. 8:209-213 (1993); and Canonico, et al., Am. J. Respir.

Cell Mol. Biol. 10:24-29 (1994)). Expression in pulmonary tissues and the endothelium was reported after intravenous injection of the complexes (Brigham, et al., Am. J. Respir. Cell Mol. Biol. 8:209-213 (1993); Zhu, et al., Science, 261:209-211 (1993); Stewart, et al., Human Gene Therapy 3:267-275 (1992); Nabel, et al., Human Gene Therapy 3:649-656 (1992); and Canonico, et al., J. Appl. Physiol. 77:415-419 (1994)). An expression cassette for an RNAi pathway sequence in linear, plasmid or viral DNA forms can be condensed through ionic interactions with the cationic lipid to form a particulate complex for *in vivo* delivery (Stewart, et al., Human Gene Therapy 3:267-275 (1992)).

Other liposome formulations, for example, proteoliposomes which contain viral envelope receptor proteins, i.e., virosomes, have been found to effectively deliver genes into hepatocytes and kidney cells after direct injection (Nicolau, et al., Proc. Natl. Acad. Sci. USA 80:1068-1072 (1993); Kaneda, et al., Science 243:375-378 (1989); Mannino, et al., Biotechniques 6:682 (1988); and Tomita, et al., Biochem. Biophys. Res. Comm. 186:129-134 (1992)).

Direct injection can also be used to administer an RNAi pathway nucleic acid sequence in a DNA expression vectors, e.g., into the muscle or liver, either as a solution or as a calcium phosphate precipitate (Wolff, et al., Science 247:1465-1468 (1990); Ascadi, et al., The New Biologist 3:71-81 (1991); and Benvenisty, et al., Proc. Natl. Acad. Sci. USA 83:9551-9555 (1986)).

20

Preparation of RNAi Agents

RNAi pathway components can be used to prepare RNAi agents. Such agents are dsRNAs that have been treated with RNAi pathway components rendering the treated dsRNA capable of activity in the RNAi pathway and can be used as sequence-specific interfering agents useful for targeted genetic interference. Specifically, treating a dsRNA with an RDE-1 and RDE-4 is useful for making an RNAi agent. An RNAi agent can be produced by preincubating a dsRNA *in vitro* in the presence of RDE-1 and RDE-4.

Another method of preparing an RNAi agent is to activate the RNAi pathway in a target cell (i.e., a cell in which it is desirable to activate the RNAi pathway such as a tumor cell) by transgenesis of an *rde-1* coding sequence and an *rde-4* coding sequence into the target cell.

30

RNAi pathway polypeptides can be modified, e.g., to enhance their stability or cellular uptake, by attaching lipophilic or other helper groups to the polypeptide, by the formation of chimeras with proteins or other moieties that are taken up by cells, or by the use of liposomes or other techniques of drug delivery known in the art.

5 In *C. elegans*, RNAi agents appear to spread from cell to cell, thus, active RNAi agents can diffuse or be actively transported from conditioned media or serum directly into target cells. Alternatively, RNAi agents can be injected into an organism or cell. They may also be incorporated into a cell using liposomes or other such methods known in the art.

10 Such methods are useful for stimulating the RNAi pathway in *C. elegans* cells, and in heterologous cells including plants and vertebrate cells. Such methods are useful in mammalian, e.g., human cells.

Enhanced Delivery of a Cargo Compound

15 RNAi pathway components that mediate the transport of dsRNA into cells and tissues can be used to promote the entry of dsRNA into cells and tissues, including dsRNA that is linked to another compound. The method is accomplished by linking dsRNA to a cargo compound (e.g., a drug or DNA molecule), e.g., by a covalent bond. The endogenous RNAi pathway gene expressing dsRNA transport function is activated
20 using methods known in the art. Alternatively, other methods can be used such as transfecting the target cell with the gene that affects transport thus permitting the cell or tissue to take up the dsDNA.

Examples

25 The invention is further described in the Examples below which describe methods of identifying mutations in the RNAi pathway and methods of identifying genes encoding components of the RNAi pathway.

Example 1: Strains and Alleles

30 The Bristol strain N2 was used as standard wild-type strain. The marker mutations and deficiencies used are listed by chromosomes as follows: LGI: *dpy-*

14(e188), *unc-13(e51)*; LGIII: *dpy-17(e164)*, *unc-32(e189)*; LGV: *dpy-11(e224)*, *unc-42(e270)*, *daf-11(m87)*, *eDf1*, *mDf3*, *nDf31*, *sDf29*, *sDf35*, *unc-76(e911)*. The *C. elegans* strain DP13 was used to generate hybrids for STS linkage-mapping (Williams et al., 1992, *Genetics* 131:609-624).

- 5 Sensitivity to RNAi was tested in the following strains. MT3126: *mut-2(r459)* (obtained from John Collins, Department of Biochemistry & Molecular Biology, University of New Hampshire, Durham, NH); *dpy-19(n1347)*, TW410: *mut-2(r459)* *sem-4(n1378)*, NL917: *mut-7(pk204)*, SS552: *mes-2(bn76)* *rol-1(e91)/mnC1* (obtained from S. Strome, Biology Dept., Indiana University), SS449: *mes-3(bn88)* *dpy-5(e61)* (from S. Strome, *supra*); *hDp20*, SS268: *dpy-11(e224)* *mes-4(bn23)* *unc-76(e911)/nT1*, SS360: *mes-6(bn66)* *dpy-20(e1282)/nT1*, CB879: *him-1(e879)*. A non-Unc *mut-6* strain used was derived from RW7096: *mut-6(st702)* *unc-22(st192::Tc1)*, due to the loss of Tc1 insertion in *unc-22*.

- 15 Homozygous mutants of *mut-6*, *mes-2*, *3*, *4*, *6* and *him-1* showed sensitivity to RNAi by injection of *pos-1* dsRNA. The dose of injected RNA was about 0.7mg/ml. This dose lies within the range where reduced concentration leads to reduced interference effects. The results of the injection of *pos-1* dsRNA into these mutants (dead embryos / F1 progeny) were as follows: *mut-6*: 422/437, *mes-2*: 781/787, *mes-3*: 462/474, *mes-4*: 810/814, *mes-6*: 900/1,002, *him-1*: 241/248, N2 (control): 365/393.

- 20 To test mutator activity, a mutant that was caused by Tc4 transposon insertion was used; TR1175: *unc-22(r765::Tc4)*. Strains TW410 and TR1175 were gifts from Q. Boese and J. Collins (Department of Biochemistry & Molecular Biology, University of New Hampshire, Durham, NH).

25 Example 2: RNA interference assay

- Genetic interference using RNAi administered by microinjection was performed as described in Fire et al., 1998, *supra* and Rocheleau et al., 1997, *Cell* 90:707-716. *pos-1* cDNA clone yk61h1, *par-2* cDNA clone yk96h7, *sqt-3* cDNA clone yk75f2 were used to prepare dsRNA *in vitro*. These cDNA clones were obtained from the *C. elegans* cDNA project (Y. Kohara, Gene Network Lab, National Institute of Genetics, Mishima 30 411, Japan).

Genetic interference using RNAi administered by feeding was performed as described in Timmons and Fire, 1998, *Nature* 395:854. *pos-1* cDNA was cloned into a plasmid that contains two T7 promoter sequences arranged in head-to-head configuration. The plasmid was transformed into an *E. coli* strain, BL21(DE3), and the transformed
5 bacteria were seeded on NGM (nematode growth medium) plates containing 60µg/ml ampicillin and 80µg/ml IPTG. The bacteria were grown overnight at room temperature to induce *pos-1* dsRNA. Seeded plates (BL21(DE3)[dsRNA] plates) stored at 4°C remained effective for inducing interference for up to two weeks. To test RNAi sensitivity, *C. elegans* larvae were transferred onto BL21(DE3)[dsRNA] plates and
10 embryonic lethality was assayed in the next generation.

Transgenic lines expressing interfering RNA for *unc-22* were engineered using a mixture of three plasmids: pPD[L4218] (*unc-22* antisense segment, driven by *myo-3* promoter); pPD[L4218] (corresponding *unc-22* sense segment, driven by *myo-3* promoter); pRF4 (semidominant transformation marker). DNA concentrations in the
15 injected mixture were 100µg/ml each. Injections were as described (Mello et al., 1991, *EMBO J.* 10:3959; Mello and Fire, 1995, *Methods in Cell Biol.* 48:451-482).

Example 3: Identification of RNAi-Deficient Mutants

A method of screening for mutants defective in the RNAi pathway was devised
20 that would permit the large-scale application of dsRNA to mutagenized populations. Feeding worms *E. coli* which express a dsRNA, or simply soaking worms in dsRNA solution, are both sufficient to induce interference in *C. elegans* (Timmons and Fire, 1998, *supra*; Tabara et al., 1998, *Science* 282:430-431). To carry out a selection, the feeding method was optimized to deliver interfering RNA for an essential gene, *pos-1*.
25 *C. elegans* hermaphrodites that ingest bacteria expressing dsRNA corresponding to a segment of *pos-1* are themselves unaffected but produce dead embryos with the distinctive *pos-1* embryonic lethal phenotype.

To identify strains defective in the RNAi pathway, wild-type animals were mutagenized, backcrossed, and the F2 generation examined for rare individuals that were
30 able to produce complete broods of viable progeny. Chemical mutagenesis was used to generate the mutations as well as spontaneous mutations arising in the *mut-6* strain in

which Tc1 transposons are activated (Mori et al., 1988, *Genetics* 120:397-407). To facilitate screens for mutations, an egg laying starting strain was used. In the absence of egg laying, the F3 progeny remained trapped within the mother's cuticle. Candidate mutants had internally hatched broods of viable embryos and were thus easily distinguished from the background population of individuals filled primarily with dead embryos (Figure 1A). Candidates were then re-tested for resistance to injected dsRNA.

The genetic screen used to isolate RNAi pathway mutants was similar to one designed by James R. Preiss for the identification of maternal effect mutants (Kemphues et al., 1988, *Cell* 52:311-320). An Egl strain, *lin-2(e1309)* was mutagenized with EMS and the F2 generation was cultured on a bacterial lawn expressing *pos-1* dsRNA. Mutagenized populations were then screened for rare individuals that were able to produce complete broods of viable progeny forming a distinctive "bag of worms" phenotype. To make sure that the animals were truly resistant to RNAi, candidate strains were next assayed for resistance to RNAi by injection. Independent EMS induced alleles of *rde-1* were found in two separate pools of mutagenized animals at a frequency of approximately one allele in 2,000 to 4,000 haploid genomes.

In addition, a search was made for spontaneous mutants using a *mut-6* strain in which Tc1 transposons are activated (Mori et al., 1988). 100,000 *mut-6; lin-2* animals (Mello et al., 1994) were cultured on bacteria expressing *pos-1* dsRNA. After one generation of growth, surviving animals were transferred again to plates with bacteria expressing the dsRNA and screened for resistant mutants. Three resulting strains were genetically mapped. One of these strains (*ne300*) mapped to LGV and failed to complement *rde-1(ne219)*. Two strains *ne299* and *ne301* mapped to LGIII and define the *rde-4* complementation group. Because the screen was clonal in nature and involved rounds of enrichment it is possible that both *rde-4* strains are related.

Seven mutant strains were selected for genetic mapping. These seven mutants defined four complementation groups; *rde-1*, with three alleles, *rde-4*, with two alleles, and *rde-2*, and *rde-3*, with one allele each (Figure 1B).

To map the RNAi defective mutations, the RNAi resistant phenotype was assayed either by feeding bacteria expressing *pos-1* dsRNA or by injection of a dsRNA mixture of *pos-1* and *unc-22*. The same assays were used for complementation tests. *In vivo*

expression of *unc-22* dsRNA was also used for mapping of *rde-1*. Mapping with visible marker mutations was performed as described in Brenner (1974, *Genetics*, 77:71-94) and mapping with STS marker was performed as described in Williams et al. (1992, *supra*).

ne219, *ne297* and *ne300* failed to complement each other, defining the *rde-1* locus. *rde-1* mutations mapped near *unc-42* V. Three factor mapping was used to locate *rde-1(ne300)* one eighth of the distance from *unc-42* in the *unc-42/daf-11* interval (3/24 Unc-non-Daf recombinants analyzed). The *rde-1(ne300)* allele complemented the chromosomal deficiency *sDf29* and failed to complement *eDf1*, *mDf3*, *nDf31* and *sDf35*. *rde-2(ne221)* and *rde-3(ne298)* mapped near *unc-13* I. *rde-2* complemented *rde-3*. *rde-4(ne299)* and *(ne301)* mapped near *unc-69* III and failed to complement each other. *ne299* complemented *mut-7(pk204)*.

The *rde-1(+)* activity is sufficient maternally or zygotically. To test the maternal sufficiency, animals heterozygous for *rde-1(ne219)* were injected with dsRNA targeting the zygotic gene, *sqt-3*, and self progeny were assayed for the Sqt phenotype. 100% of the self progeny including *rde-1* homozygous progeny were found to exhibit the Sqt phenotype. Thus, maternally provided *rde-1(+)* activity is sufficient to mediate interference with a zygotic target gene. Zygotic sufficiency was assayed by injecting homozygous *rde-1* mothers with dsRNA targeting the zygotic *unc-22* gene (Figure 3). Injected animals were allowed to produce self-progeny or instead were mated after 12 hours to wild-type males, to produce heterozygous *rde/+* cross-progeny. Each class of progeny was scored for the *unc-22* twitching phenotype as indicated by the fraction shown in Figure 3 (Unc progeny/total progeny). The injected animals were then mated with wild-type males. Self progeny from homozygous injected mothers were unaffected, however, 68% of the cross progeny were Unc. This result indicates that zygotically provided *rde-1(+)* activity is also sufficient. However both maternal and zygotic *rde-1(+)* activity contribute to zygotic interference as 100% of progeny from wild-type injected mothers exhibit *unc-22* interference (606/606). Thus, *rde-1(+)* and *rde-4(+)* activities are not needed for dsRNA uptake, transport or stability.

RNAi sensitivity of several existing *C. elegans* mutants was also examined. Most of these mutant strains were fully sensitive to RNAi. However, RNAi resistance was identified in two strains that had previously been shown to exhibit elevated levels of

transposon mobilization (mutator strains): *mut-2* (described in Collins et al., 1987, *Nature* 328:726-728) and *mut-7* (described in Ketting et al., Cell, in press for release on October 15, 1999). Another mutator strain, *mut-6(st702)*, was fully sensitive to RNAi. Since mutator strains continually accumulate mutations, the resistance of *mut-2* and *mut-7* may have been due to the presence of secondary mutations. To test this possibility we examined the genetic linkage between the mutator and RNAi resistance phenotypes of *mut-2* and *mut-7*. We found that independently outcrossed *mut-2(r459)* mutator strains TW410 and MT3126 both showed resistance to RNAi. We mapped the RNAi resistance phenotype of *mut-7(pk204)* to the center of linkage group III (Figure 1B), the position that had been defined for the mutator activity of *mut-7(pk204)* by Ketting et al. (*supra*). Together, these observations indicate that the RNAi resistance phenotypes of the *mut-2* and *mut-7* strains are genetically linked to their mutator activities. Animals heterozygous for the *rde* and *mut* alleles were generated by crossing wild-type males with Unc-Rde or Unc-Mut hermaphrodites. The *rde* and *mut* mutations appeared to be simple recessive mutations with the exception of *mut-2(r459)*, which appeared to be weakly dominant (Figure 2A).

These data demonstrate that some genes are non-essential (e.g., *rde-1* and *rde-4*).

This method can be used to identify additional mutations in RNAi pathway genes.

Example 4: Identification of Properties of RNAi-Deficient Mutants

Effects of rde mutations in germline and somatic tissues

Microinjection was used to assay the sensitivity of each *rde* strain to several distinct dsRNA species. The *pos-1* and *par-2* genes are expressed in the maternal germline and are required for proper embryonic development (Tabara et al., 1999, *Development* 126:1-11; Boyd et al., 1996, *Development* 122:3075-3084). All *rde*- strains tested (as well as *mut-2* and *mut-7*) showed significant resistance to dsRNA targeting of these germline-specific genes (Figure 2B), as well as to several other germline specific genes tested. The *rde-3* data (asterisk in Figure 2B) includes a 10% non-specific embryonic lethality present in the *rde-3* strain.

To examine the effect of these mutations on genetic interference of somatically expressed genes, cells were injected with dsRNA targeting the cuticle collagen gene *sqt-3*

and the body muscle structural gene *unc-22*. *sqt-3* hypomorphic mutants exhibit a short, dumpy body shape (dpy; van der Keyl et al., 1994, *Dev. Dyn.* 201:86-94). *unc-22* mutations exhibit severe paralysis with a distinctive body twitching phenotype (Moerman et al., 1986, *Proc. Natl. Acad. Sci. USA* 83:2579-2583). *rde-1*, *rde-3*, *rde-4* and *mut-2* strains showed strong resistance to both *sqt-3* and *unc-22* dsRNA, while *rde-2* and *mut-7* strains showed partial resistance. Thus *rde-2* and *mut-7* appeared to be partially tissue- or gene-specific in that they were required for effective RNAi against germline but not somatically expressed genes. The *rde-1*, *rde-3*, *rde-4*, and *mut-2* (+) activities appeared to be required for interference for all genes analyzed. The *rde* and *mut* strains differ from one another in sensitivity to *sqt-2* dsRNA.

Effect of rde on transposon mobilization

The effect of *rde* mutations on transposon mobilization was examined. Two of the newly identified mutants, *rde-2* and *rde-3* exhibited a level of transposon activation similar to that of *mut-7* (Table 1). In contrast, transposon mobilization was not observed in the presence of *rde-1* or *rde-4* (Table 1).

TABLE 1: TRANSPOSON MOBILIZATION AND MALE INCIDENCE IN *rde* AND *mut* STRAINS

Percentage of Non-Unc		Revertants
unc-22 (r765::Tc4)		0 (0/2000)
rde-1 (ne219); unc-22 (r765::Tc4)		0 (0/4000)
rde-2 (ne221); unc-22 (r765::Tc4)		0.96 (8/830)
rde-3 (ne298); unc-22 (r765::Tc4)		1.6 (35/2141)
rde-4 (ne299); unc-22 (r765::Tc4)		0 (0/2885)
mut-7 (pk204); unc-22 (r765::Tc4)		1.0 (40/3895)
Percentage of Male Animals		
Wild type (n2)		0.21 (2/934)
rde-1 (ne219)		0.07 (1/1530)
rde-2 (ne221)		3.2 (25/788)
rde-3 (ne298)		7.8 (71/912)
rde-4 (ne299)		0.24 (5/2055)

5

X-chromosome loss

Mutator strains (including *mut-2*, *mut-7*) *rde-2* and *rde-3*) exhibit a second phenotype: a high incidence of males reflecting an increased frequency of X-chromosome loss during meiosis (Collins et al., 1987, *supra*; Ketting et al., *supra*). This

phenotype was observed in *rde-2* and *rde-3* strains, but not observed in the *rde-1* and *rde-4* strains which showed a wild-type incidence of males (Table 1).

A previously described gene-silencing process appears to act on transgenes in the germline of *C. elegans*. Although the silencing mechanisms are not well understood, they are known to depend on the products of the genes *mes-2*, *3*, *4* and *6* (Kelly and Fire, 1998, *Development* 125:2451-2456). To examine the possibility that the RNAi and germline transgene-silencing might share common mechanistic features, we first asked if the *mes* mutants were resistant to RNAi. We found normal levels of RNA interference in each of these strains. We next asked if RNAi deficient strains were defective in transgene-silencing. Three strains were analyzed: *mut-7(pk204)*, *rde-1(ne219)* and *rde-2(ne221)*.

To analyze transgene silencing in *mut-7* worms, homozygous *mut-7* lines carrying various GFP reporters transgenes were generated as follows: N2 (Bristol strain) males were mated to *mut-7(pk204) unc-32(e189)* hermaphrodites; cross progeny males were then mated to strains carrying the GFP transgenes. *mut-7 unc-32/++* cross progeny from these matings were cloned, and *mut-7 unc-32* homozygous animals carrying the transgenes were isolated from their self-progeny. After the GFP reporter transgenes were introduced into different genetic backgrounds, activation of GFP transgene expression in germ cells was assayed at 25°C by fluorescence microscopy. The tested GFP reporter transgenes were each active in some or all somatic tissues, but had become silenced in the germline. The plasmids used and transgene designations are as follows: 1) pBK48 which contains an in-frame insertion of GFP into a ubiquitously expressed gene, *let-858* (Kelly, et al., 1997, *Genetics* 146:227-238). *ccExPD7271* contains more than 100 copies of pBK48 in a high copy repetitive array that is carried extrachromosomally. 2) pJH3.92 is an in-frame fusion of GFP with the maternal *pie-1* gene (M. Dunn and G. Seydoux, Johns Hopkins University, Baltimore, MD). *jhEx1070* carries pJH3.92 in a low copy "complex" extrachromosomal array generated by the procedure of Kelly et al. (1997, *supra*) pJKL380.4 is a fusion of GFP with the *C. elegans* nuclear laminin gene, *lam-1*, which is expressed in all tissues (J. Liu and A. Fire). *ccIn4810* carries pJKL380.4 in a complex array that has been integrated into the X chromosome by gamma irradiation using standard techniques.

The *mut-7* strain was analyzed most extensively and was found to exhibit desilencing of three different germline transgenes tested (Table 2). The *rde-2* strain exhibited a similar level of desilencing for a single transgene. In contrast, no transgene desilencing was observed in *rde-1* mutants (Table 2). Thus, *mut-7* and *rde-2* which differ from *rde-1* in having transposon mobilization and a high incidence of X-chromosome loss also differ from *rde-1* in their ability to partially reactivate silent germline transgenes.

10 **TABLE 2: REACTIVATION OF SILENCED TRANSGENES IN THE GERMLINE OF *mut-7(pk204)***

Genotype	Transgene Array	Percentage of Germline Desilencing
+/+	ccEx7271	8.3 (4/48)
mut-7/+	ccEx7271	14.5 (7/48)
mut-7/mut-7	ccEx7271	91.0 (71/78)
+/+	jhEx1070	3.9 (2/51)
mut-7/mut-7	jhEx1070	86.5 (32/37)
+/+	ccin4810	4.3 (2/46)
mut-7/mut-7	ccin4810	73.3 (33/45)
rde-1/rde-1	ccEx7271	0 (0/34)

15 **Example 5: Requirement for *rde-1*(+) and *rde-4*(+) Activities in Target Tissue**

The *rde-1* and *rde-4* mutants differ from other RNAi deficient strains identified herein in that they do not cause transposon mobilization nor do they cause chromosome

loss. The role of these genes in upstream events such as dsRNA uptake, transport or stability was examined. Such events could be required for interference induced by exogenous trigger RNAs but might be dispensable for natural functions of RNAi. To evaluate these upstream events, *rde-1* and *rde-4* homozygotes were exposed to dsRNA. The next generation was scored for interference. dsRNA targeting the *unc-22* gene was injected into the intestinal cells of homozygous *rde-1* and *rde-4* hermaphrodites and the injected animals were then mated to wild-type males (Figure 3). The self-progeny for both strains exhibited no interference with the targeted gene. However, there was potent interference in the *rde-1/+* and *rde-4/+* cross progeny (Figure 3). These observations indicated that *rde-1* and *rde-4* mutants have intact mechanisms for transporting the interference effect from the site of injection (the intestine) into the embryos of the injected animal and then into the tissues of the resulting progeny. The stability of the resulting interference also appeared to be normal in *rde-1* and *rde-4* as the homozygous injected mothers continued to produce affected cross progeny for several days after the time of injection.

To examine whether *rde-1* and *rde-4* mutants could block interference caused by dsRNA expressed directly in the target tissue, the muscle-specific promoter from the *myo-3* gene (Dibb et al., 1989, *J. Mol. Biol.* 205:605-613) was used to drive the expression of both strands of the muscle structural gene *unc-22* in the body wall muscles (Moerman et al., 1986, *supra*; Fire et al., 1991, *Development* 113:503-514). A mixture of three plasmids was injected: [*myo-3* promoter::*unc-22* antisense], [*myo-3*::*unc-22* sense], and a marker plasmid (pRF4[rol-6(su1006gf)] [Mello et al., 1991]). Frequencies of *Unc* transgenic animals were followed in F1 and F2 generations. The ^a*Unc* phenotype was weak. Wild-type animals bearing this transgene exhibit a strong twitching phenotype consistent with *unc-22* interference. The twitching phenotype was strongly suppressed by both *rde-1* and *rde-4* mutants (Table 3). The *mut-7* and *rde-2* mutants which are both sensitive to *unc-22(RNAi)* by microinjection were also sensitive to promoter driven *unc-22* interference in the muscle (Table 3). Taken together these findings suggest that *rde-1(+)* and *rde-4(+)* activities are not necessary for uptake or stability of the interfering RNA and may function directly in the target tissue.

TABLE 3: SENSITIVITY OF *rde* AND *mut* STRAINS TO TRANSGENE-DRIVEN INTERFERING RNA

Unc Animals in Transgenic F1	Unc F2 Lines in Inherited Lines	
Wild type (N2)	26/59	10/11
<i>rde-1</i> (ne219)	0/25	0/3
<i>rde-2</i> (ne221)	35/72	14/14
<i>rde-3</i> (ne298)	1 ^a /38	1 ^a /9
<i>rde-4</i> (ne299)	0/51	0/4
<i>mut-7</i> (pk204)	9/13	3/3

5

Example 6: Molecular Identification of the *rde-1* Gene

The *rde-1* gene was cloned using standard genetic mapping to define a physical genetic interval likely to contain the gene using YACs and cosmids that rescue *rde-1* mutants. These were used to identify a cloned *rde-1* cDNA sequence and a cloned *rde-4* sequence. These methods can also be used to identify the genes for *rde-2*, *rde-3*, and *rde-5* using the mutant strains provided herein.

To clone an *rde-1* gene, yeast artificial chromosome clones (YACs) containing *C. elegans* DNA from this interval were used to rescue the *rde-1* mutant phenotype. To facilitate this analysis candidate rescuing YACs were co-injected with plasmids designed to express *unc-22*(RNAi). YAC and cosmid clones that mapped near the *rde-1* locus were obtained from A. Coulson. *rde-1*(ne219) was rescued by YAC clones: Y97C12 and Y50B5. The two overlapping YAC clones provided *rde-1* rescuing activity as indicated by *unc-22* genetic interference with characteristic body paralysis and twitching in the F1 and F2 transgenic animals. In contrast a non-overlapping YAC clone failed to rescue resulting in 100% non-twitching transgenic strains (Figure 4A).

The rescuing activity was further localized to two overlapping cosmid clones, cosmid C27H6 and T10A5, and finally to a single 4.5kb genomic PCR fragment predicted to contain a single gene, designated K08H10.7 (SEQ ID NO:1; Figures 5A-5C). The K08H10.7 PCR product gave strong rescue when amplified from wild-type genomic DNA. This rescue was greatly diminished using a PCR fragment amplified from any of the three *rde-1* alleles and was abolished by a 4 bp insertion at a unique *NheI* site in the *rde-1* coding region. A wild-type PCR product from an adjacent gene C27H6.4, also failed to rescue.

The K08H10.7 gene from each of the *rde-1* mutant strains was sequenced, and distinct point mutations were identified that are predicted to alter coding sequences in K08H10.7 (Figure 4A). Based on these findings *rde-1* can be identified as the K08H10.7 gene.

A full-length cDNA sequence was determined for *rde-1* using the cDNA clones, yk296b10 and yk595h5. cDNA clones for *rde-1* were obtained from Y. Kohara (Gene Network Lab, National Institute of Genetics, Mishima 411, Japan). The cDNA sequence of coding region and 3'UTR was determined on yk296b10 except that the sequence of 5'UTR was determined on yk595h5. The GenBank accession number for *rde-1* cDNA is AF180730 (SEQ ID NO:2). The *rde-1* cDNA sequence was used to generate a predicted translation product (SEQ ID NO:3), referred to as RDE-1, consisting of 1020 amino acids. The RDE-1 sequence was used to query Genbank and identify numerous related genes in *C. elegans* as well as other animals and plants. This gene family includes at least 23 predicted *C. elegans* genes, several of which appear to be members of conserved subfamilies. Within subfamilies, conservation extends throughout the protein and all family members have a carboxy-terminal region that is highly conserved (Figure 4B). Besides the genes shown in Figure 4B, other related genes include ARGONAUTE 1 (*Arabidopsis*), SPCC736.11 (*S. pombe*), and Piwi (*Drosophila*). A portion of the N terminal region of RDE-1 showed no significant similarity to any of the identified related genes. There are no defined functional motifs within this gene family, but members including RDE-1 are predicted to be cytoplasmic or nuclear by PSORT analysis (Nakai and Horton, 1999, *Trends Biochem. Sci.* 24:34-36). Furthermore, one family member named eIF2C has been identified as a component of a cytoplasmic

protein fraction isolated from rabbit reticulocyte lysates. The RDE-1 protein is most similar to the rabbit eIF2C. However, two other *C. elegans* family members are far more similar to eIF2C than is RDE-1 (Figure 4B). RDE-1 may provide sequence-specific inhibition of translation initiation in response to dsRNA.

5 The *rde-1* mutations appear likely to reduce or eliminate *rde-1(+)* activity. Two *rde-1* alleles *ne219* and *ne297* are predicted to cause amino acid substitutions within the RDE-1 protein and were identified at a frequency similar to that expected for simple loss-of-function mutations. The *rde-1(ne219)* lesion alters a conserved glutamate to a lysine (Figure 4B). The *rde-1(ne297)* lesion changes a non-conserved glycine, located four
10 residues from the end of the protein, to a glutamate (Figure 4B). The third allele, *ne300*, contains the strongest molecular lesion and is predicted to cause a premature stop codon prior to the most highly conserved region within the protein (Q>Ochre in Figure 4B). Consistent with the idea that *rde-1(ne300)* is a strong loss of function mutation, we found that when placed in trans to a chromosomal deficiency the resulting deficiency trans-
15 heterozyotes were RNAi deficient but showed no additional phenotypes. These observations suggest that *rde-1* alleles are simple loss-of-function mutations affecting a gene required for RNAi but that is otherwise non-essential.

Because of its upstream role RNA interference (see Examples 8-10 below), the RDE-1 protein and fragments thereof can be used to prepare dsRNA that is useful as an
20 RNAi agent.

Example 7: Maternal Establishment and Paternal Transmission of RNAi

To examine whether the interference effect induced by RNAi exhibited linkage to the target gene (e.g., was involved in a reversible alteration of the gene or associated
25 chromatin), a strain was constructed such that the F1 males that carry the RNAi effect also bear a chromosomal deletion that removes the target gene (Fig. 7B). In the case of linkage to the target gene, the RNAi effect would be transmitted as a dominant factor.

In experiments testing the linkage of the interference effect to the target gene, three different species of dsRNA (*pos-1* dsRNA, *mom-2* dsRNA, or *sgg-1* dsRNA) were
30 delivered into *C. elegans* in independent experiments. The dsRNA was delivered by injection through a needle inserted into the intestine. In general, dsRNA was synthesized

in vitro using T3 and T7 polymerases. Template DNA was removed from the RNA samples by DNase treatment (30 minutes at 37°C). Equal amounts of sense and antisense RNAs were then mixed and annealed to obtain dsRNA. dsRNA at a concentration of 1-5 mg/ml was injected into the intestine of animals. In control experiments, mixtures of linearized template DNA plasmids used for synthesizing RNA failed to induce interference in P0, F1, or F2 animals when injected into the intestine of hermaphrodites at a concentration of 0.2 mg/ml. Fig. 7A illustrates this experiment. The gonad of the parent (P0) hermaphrodite has symmetrical anterior and posterior U-shaped arms as shown in Fig. 7A. Several fertilized eggs are shown in Fig. 7A, centrally located in the uterus. The rectangular mature oocytes are lined up in the gonad arms most proximal to the uterus. The embryos present in P0 at the time of injection gave rise to unaffected F1 progeny. Oocytes in the proximal arms of the injected P0 gonad inherit the RNAi effect but also carry a functional maternal mRNA (F1 carriers of RNAi).

After a clearance period during which carrier and unaffected F1 progeny are produced, the injected P0 begins to exclusively produce dead F1 embryos with the phenotype corresponding to the inactivation of the gene targeted by the injected RNA (Tabara et al. 1999, Development 126:1; C. Rocheleau, 1997, Cell 90:707). Potential F1 and F2 carriers of the interference effect were identified within the brood of the injected animal. In the case of hermaphrodites, carriers were defined as "affected" if the animals produced at least 20% dead embryos with phenotypes corresponding to maternal loss of function for the targeted locus. In the case of males, carriers were defined as animals whose cross progeny included at least one affected F2 hermaphrodite. The total number of carriers identified in each generation for each of three dsRNAs injected is shown in Fig. 7A as a fraction of the total number of animals assayed.

To examine the extragenic inheritance of RNAi, experiments were carried out investigating whether sperm that inherit the deletion and therefore have no copies of the target locus could carry the interference effect into the F2 generation. F1 males that carried both *pos-1* (RNAi) and a chromosomal deficiency for the *pos-1* locus were generated. The chromosome carrying the deficiency for *pos-1* also carried a deficiency for phenotypically uncoordinated (*unc*). F2 progeny of the carrier male includes two genotypes: phenotypically wild-type animals that inherit the (+) chromosome, and

phenotypically uncoordinated (Unc) progeny that inherit the mDf3 chromosome. In these experiments, the deficiency bearing sperm were just as capable as wild-type sperm of transferring interference to the F2 hermaphrodite progeny (Fig. 7B). Thus, the target locus was not needed for inheritance of the interference effect.

5 Surprisingly, although males were sensitive to RNAi and could inherit and transmit RNAi acquired from their mothers, direct injections into males failed to cause transmission of RNAi to the F1 for several genes tested. In an example of this type of protocol, wild type males were injected with targeting dsRNA: body muscle structural gene *unc-22*, cuticle collagen gene *sqt-3*, maternal genes *pos-1* and *sgg-1*. Males of the
 10 *pes-10::gfp* strain (Seydoux, G. and Dunn, MA, 1997, Development 124:2191-2201) were injected with *gfp* dsRNA. Injected males were affected by *unc-22* and *gfp* dsRNA to the same extent as injected hermaphrodites. No RNAi interference was detected in F1 progeny or injected males (40 to 200 F1 animals scored for each RNA tested). Therefore, the initial transmission of RNAi to F1 progeny may involve a mechanism active only in
 15 hermaphrodites while subsequent transmission to the F2 progeny appears to involve a distinct mechanism, active in both hermaphrodites and males. The hermaphrodite-specific step may indicate the existence of a maternal germline process that amplifies the RNAi agent. These data show that extracts from the maternal germline tissues of *C. elegans* may be used in conjunction with RDE-1 and RDE-4 activity to create and to then
 20 amplify RNAi agents.

In addition, the germline factors that amplify the RNAi agents can be identified by mutations that result in an RNAi deficient mutant phenotype. Such factors can be used as additional components of an *in vitro* system for the efficient amplification of RNAi agents.

25

Example 8: Sufficiency of Wild-Type Activities of *rde-1*, *rde-2*, *mut-7*, and *rde-4* in Injected Animals for Interference Among F1 Self Progeny

To investigate whether the activities of *rde-1*, *rde-2*, *rde-4*, and *mut-7*, respectively, are sufficient in injected hermaphrodites for interference in the F1 and F2
 30 generations, crosses were designed such that wild-type activities of these genes would be present in the injected animal but absent in the F1 or F2 generations. To examine inheritance in the F1 generation, (hermaphrodite) mothers heterozygous for each mutant

(P0) were injected, allowed to produce self-progeny (F1) and the homozygous mutant progeny in the F1 generation were examined for genetic interference (Fig. 8A). To do this, the heterozygous hermaphrodites from each genotype class, *rde-1*, *unc-42/+*, *rde-2*, *unc-13/+*, *mut-7*, *dpy-17/+*; and *rde-4*, *unc-69/+* (the following alleles were used in this study: *rde-1(ne300)* *unc-42*, *rde-1(ne219)*, *rde-2(ne221)*, *rde-4(ne299)*, and *mut-7(pk2040)*) were injected with *pos-1* dsRNA. In each case, two types of F1 self progeny, distinguished by the presence of the linked marker mutations, were scored for interference (Fig. 8A). In these experiments the *rde-1* and *rde-4* mutant F1 progeny exhibited robust interference, comparable to wild-type, while the *rde-2* and *mut-7* F1 progeny failed to do so. In control experiments, homozygous F1 progeny from heterozygous (uninjected) mothers were directly injected with *pos-1* dsRNA (Fig. 8B). Injection of dsRNA directly into the *rde-1* and *rde-4* mutant progeny of uninjected heterozygous mothers failed to result in interference. Thus, injection of dsRNA into heterozygous hermaphrodites resulted in an inherited interference effect that triggered gene silencing in otherwise RNAi resistant *rde-1* and *rde-4* mutant F1 progeny while *rde-2* and *mut-7* mutant F1 progeny remained resistant.

In this experiment, the expression of *rde-1*(+) and *rde-4*(+) in the injected animal was sufficient for interference in later generations.

These data suggest that treatment of a dsRNA with functional *rde-1* and *rde-4* gene products can produce an agent that activates the remainder of the RNAi pathway.

Example 9: Requirements for *rde-1*, *rde-2*, *rde-4*, and *mut-7* in F1 and F2 interference

To examine the genetic requirements for RNAi genes in the F2 generation, F1 male progeny were generated that carry the interference effect as well as one mutant copy of each respective locus; *rde-1*, *rde-2*, and *mut-7* (Fig. 9A). Each of these males was then backcrossed with uninjected hermaphrodites homozygous for each corresponding mutant (Fig. 9A). The resulting cross progeny (F1) included 50% heterozygotes and 50% homozygotes that were distinguished by the presence of the linked marker mutations. The heterozygous siblings served as controls and in each case exhibited interference at a frequency similar to that seen in wild-type animals (Fig. 9A). In these crosses, *rde-2* and *mut-7* homozygous F2 progeny failed to exhibit interference, indicating that the activities

of these two genes are required for interference in the F2 generation. In contrast, we found that homozygous *rde-1* F2 animals exhibited wild-type levels of F2 interference (Fig 9A). Control *rde-1* homozygotes generated through identical crosses were completely resistant to *pos-1::RNAi* when challenged *de novo* with dsRNA in the F2 generation. In these experiments, 35 *rde-1* homozygous animals generated through crosses shown in Fig. 9A were tested by feeding bacteria expressing *pos-1* dsRNA, and 21 similar animals were tested by direct injections of *pos-1* dsRNA. All animals tested were resistant to *pos-1* (RNAi). Thus, *rde-1* activity in the preceding generations was sufficient to allow interference to occur in *rde-1* mutant F2 animals while the wild-type activities of *rde-2* and *mut-7* were required directly in the F2 animals for interference.

In this experiment, the expression of *rde-1*(+) and *rde-4*(+) in the injected animal was sufficient for interference in later generations. The wild-type activities of the *rde-2* and *mut-7* genes were required for interference in all generations assayed. Thus, *rde-2* and *mut-7* might be required only downstream or might also function along with *rde-1* and *rde-4*.

These data lend additional support to the concept that an appropriately treated dsRNA could be used as an RNAi agent.

Example 10: Sufficiency of *rde-1* Activity to Initiate RNA Interference in Injected Animals That Lack the Wild-Type Activities of *rde-2*, *mut-7*, or *rde-4*

To ask if *rde-2* and *mut-7* activities function along with or downstream of *rde-1*, genetic cross experiments were designed in which the activities of these genes were present sequentially (Fig. 9B). For example, *rde-1*(+);*rde-2*(-) animals were injected with *pos-1* dsRNA and then crossed to generate F1 hermaphrodites homozygous for *rde-1*(-); *rde-2*(+). In these experiments *rde-1*(+) activity in the injected animals was sufficient for F1 interference even when the injected animals were homozygous for *rde-2* or *mut-7* mutations (Fig. 10B). In contrast, *rde-1*(+) activity in the injected animals was not sufficient when the injected animals were homozygous for *rde-4* mutant (Fig. 10B). Thus, *rde-1* can act independently of *rde-2* and *mut-7* in the injected animal, but *rde-1* and *rde-4* must function together. These findings are consistent with the model that *rde-1* and *rde-4* function in the formation of the inherited interfering agent (i.e., an RNAi agent) while *rde-2* and *mut-7* function at a later step necessary for interference.

In summary, the above Examples provide genetic evidence for the formation and transmission of extragenic interfering agents in the *C. elegans* germline. Two *C. elegans* genes, *rde-1*, and *rde-4*, appear to be necessary for the formation of these extragenic agents but not for interference mediated by them. In contrast, the activities of two other
 5 genes, *rde-2* and *mut-7*, are required only downstream for interference.

These examples provide evidence that the *rde-1* and *rde-4* gene products or their homologs (e.g., from a mammal) can be used to prepare agents effective in activating the RNAi pathway.

10 Example 11: *rde-4* Sequences

An *rde-4* gene was cloned using methods similar to those described in Example 6. The nucleic acid sequence (SEQ ID NO:4) and predicted amino acid sequence (SEQ ID NO:5) are illustrated in Fig. 10.

Analysis of the *rde-4* nucleic acid sequence shows that it encodes a protein (RDE-
 15 4) with similarities to dsRNA binding proteins. Examples of the homology to X1RBPA (SEQ ID NO:6; Swissprot: locus TRBP_XENLA, accession Q91836; Eckmann and Jantsch, 1997, J. Cell Biol. 138:239-253) and HSPKR (SEQ ID NO:7; AAF13156.1; Xu and Williams, 1998, J. Interferon Cytokine Res. 18:609-616), and a consensus sequence (SEQ ID NO:8) are shown in Fig. 11. Three regions have been identified within the
 20 predicted RDE-4 protein corresponding to conserved regions found in all members of this dsRNA binding domain family. These regions appear to be important for proper folding of the dsRNA binding domain. Conserved amino acid residues, important for interactions with the backbone of the dsRNA helix, are found in all members of the protein family including RDE-4 (see consensus residues in Figure 11). This motif is
 25 thought to provide for general non-sequence-specific interactions with dsRNA. The RDE-4 protein contains conserved protein folds that are thought to be important for the assembly of the dsRNA binding domain in this family of proteins. Conserved amino acid residues in RDE-4 are identical to those that form contacts with the dsRNA in the crystal structure of the X1RBP dsRNA complex. These findings strongly suggest that RDE-4 is
 30 likely to have dsRNA binding activity.

Because RDE-4 contains a motif that is likely to bind in a general fashion to any

dsRNA and because RDE-4 appears to function upstream in the generation of RNAi agents, the RDE-4 protein or fragments thereof can be used to convert any dsRNA into an RNAi agent. In addition to the dsRNA binding domain, RDE-4 contains other functional domains that may mediate the formation of RNAi agents. These domains may provide for interaction between RDE-4 and RDE-1 or for binding to enzymes such as nucleases that convert the dsRNA into the RNAi agent. Because of its RNA binding function in RNA interference, the RDE-4 protein and fragments thereof can be used to prepare dsRNA that is useful in preparing an RNAi agent.

10 Example 12: Identification of Regions of RDE-1 and RDE-4 that are Required for Creating an RNAi Agent

In vivo and *in vitro* assays are used to identify regions in RDE-1 and RDE-4 that are important for the generation of RNAi agents. In the *in vivo* assay, *rde-1* and *rde-4* are introduced into the corresponding *C. elegans* mutant strains via transgenes (Tabara et al., Cell 99:123 (1999); and Example 13). Important functional domains in RDE-1 and RDE-4 are defined by systematically altering the proteins followed by reintroduction into mutant animals to test for rescue of the RNAi deficient phenotype. A series of nested deletions are analyzed for rescue activity for both *rde-1* and *rde-4*. Specific point mutations are used to analyze the importance of specific amino acids. Chimera's are produced between RDE proteins and related proteins and genes. For example, coding sequences from RDE-1 homologs from the worm or from human are tested for their ability to rescue *rde-1* mutants. Replacing the RDE-4 dsRNA binding motif with a distinct RNA binding motif, e.g., one that recognizes a specific viral dsRNA sequence or a ssRNA sequence will alter the specificity of the RNAi response perhaps causing sequence-specific or ssRNA-induced gene targeting. In one form of the *in vitro* assay, whole protein extracts from *rde-1* or *rde-4* deficient worm strains are used.

Recombinant RDE-1 or RDE-4 is then added back to reconstitute the extract. Altered RDE-1 and RDE-4 proteins (described above, including deletions, point mutants and chimeras) are made *in vitro* and then tested for their ability to function when added back to these extracts. RNAi activity is analyzed by injecting the reconstituted extracts directly into animals or by assaying for the destruction of an added *in vitro* synthesized

target mRNA.

Example 13: Rescue of rde-4 Animals

Rescue of animals (e.g., *C. elegans*) that are mutant for an RNAi pathway is a
5 useful method for identifying sequences from RNAi pathway genes that encode
functional polypeptides, e.g., polypeptides that can eliminate the mutant phenotype.

An example of such a method for identifying rde-4 mutant animals is as follows.
PCR using primers located 1 kb upstream and 500 nucleotides downstream of the open
reading frame (T20G5.11; illustrated in Fig. 12) are used to amplify the rde-4 gene from
10 *C. elegans* genomic DNA. The resulting PCR product is then injected along with reporter
constructs described in Tabara et al. (Cell 99:123 (1999); incorporated herein in its
entirety by reference), and the progeny of the injected animal are assayed for rescue of
the RNAi deficient phenotype. The PCR product can also be cloned into a plasmid
vector for site directed mutational analysis of RDE-4 (see Example 12). Co-injection of
15 such a wild type RDE-4 plasmid and altered derivatives can be used to identify functional
domains of rde-4. Similar methods can be used to identify functional domains of rde-1
and other RNAi pathway components.

Other Embodiments

20 It is to be understood that while the invention has been described in conjunction
with the detailed description thereof, the foregoing description is intended to illustrate
and not limit the scope of the invention, which is defined by the scope of the appended
claims. Other aspects, advantages, and modifications are within the scope of the
following claims.

25

What is claimed is:

- 1 1. An isolated nucleic acid molecule comprising a nucleotide sequence encoding
2 an RDE-1 polypeptide, wherein the nucleic acid molecule hybridizes under high
3 stringency conditions to the nucleic acid sequence of Genbank Accession No. AF180730
4 (SEQ ID NO:2) or its complement, or nucleic acid sequence set forth in SEQ ID NO:1 or
5 its complement.
- 1 2. The isolated nucleic acid of claim 1, wherein the nucleic acid can complement
2 an rde-1 mutation.
- 1 3. An isolated nucleic acid of claim 1, wherein the nucleotide sequence encodes
2 the amino acid sequence of SEQ ID NO:3.
- 1 4. A substantially pure RDE-1 polypeptide encoded by the isolated nucleic acid
2 of claim 1.
- 1 5. An antibody that specifically binds to an RDE-1 polypeptide.
- 1 6. A method of enhancing the expression of a transgene in a cell, the method
2 comprising decreasing activity of the RNAi pathway.
- 1 7. The method of claim 6, wherein rde-2 expression or activity is decreased.
- 1 8. An isolated nucleic acid molecule comprising a nucleotide sequence encoding
2 an RDE-4 polypeptide, wherein the nucleic acid molecule hybridizes under high
3 stringency conditions to the nucleic acid sequence of SEQ ID NO:4 or its complement.
- 1 9. The isolated nucleic acid of claim 8, wherein the nucleic acid can complement
2 an rde-4 mutation.
- 1 10. An isolated nucleic acid of claim 8, wherein the nucleotide sequence encodes
2 the amino acid sequence of SEQ ID NO:5.
- 1 11. A substantially pure RDE-4 polypeptide encoded by the isolated nucleic acid
2 of claim 8.
- 1 12. An antibody that specifically binds to an RDE-4 polypeptide.
- 1 13. A method of preparing an RNAi agent, the method comprising incubating a
2 dsRNA in the presence of an RDE-1 protein and an RDE-4 protein.

1 14. A method of inhibiting the activity of a gene, the method comprising
2 introducing an RNAi agent into a cell, wherein the dsRNA component of the RNAi agent
3 is targeted to the gene.

1 15. The method of claim 14, wherein the cell contains exogenous RNAi
2 sequences.

1 16. The method of claim 14, wherein the exogenous RNAi sequence is an RDE-1
2 polypeptide or an RDE-4 polypeptide.

1/21

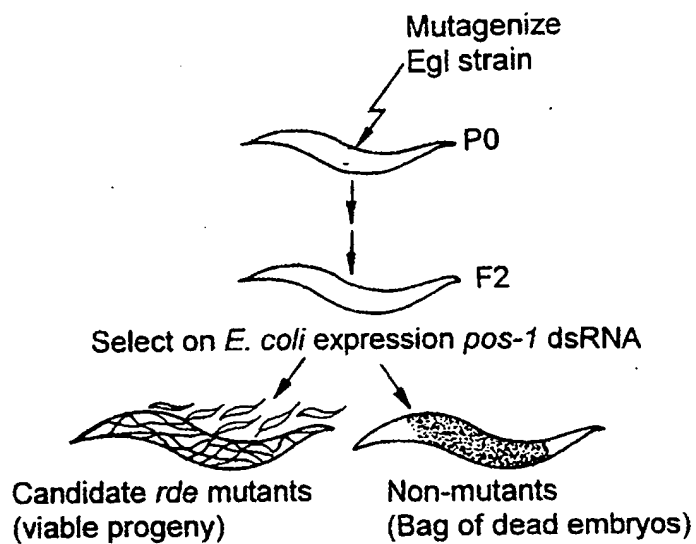


FIG. 1A

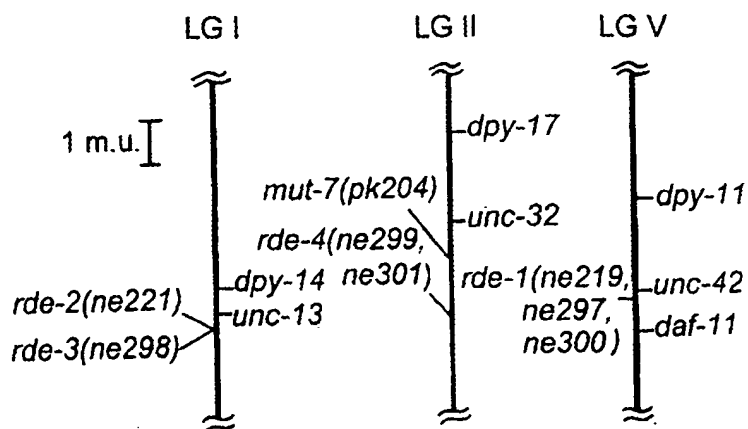


FIG. 1B

2/21

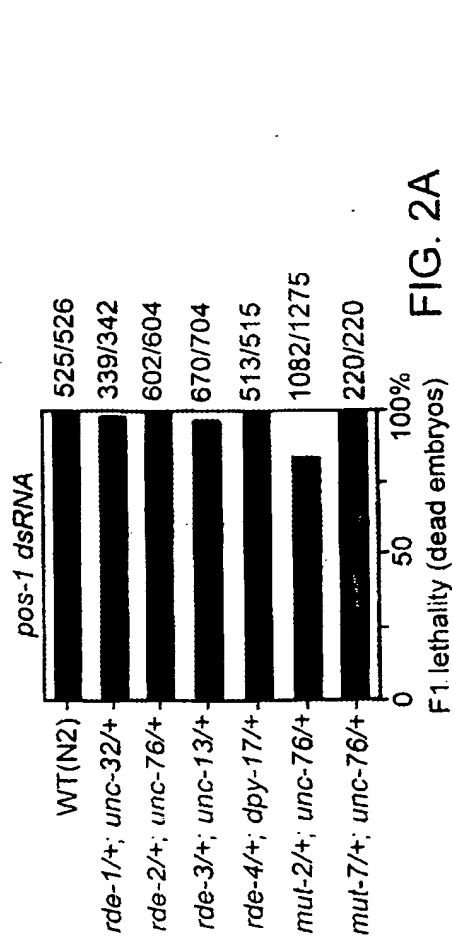


FIG. 2A

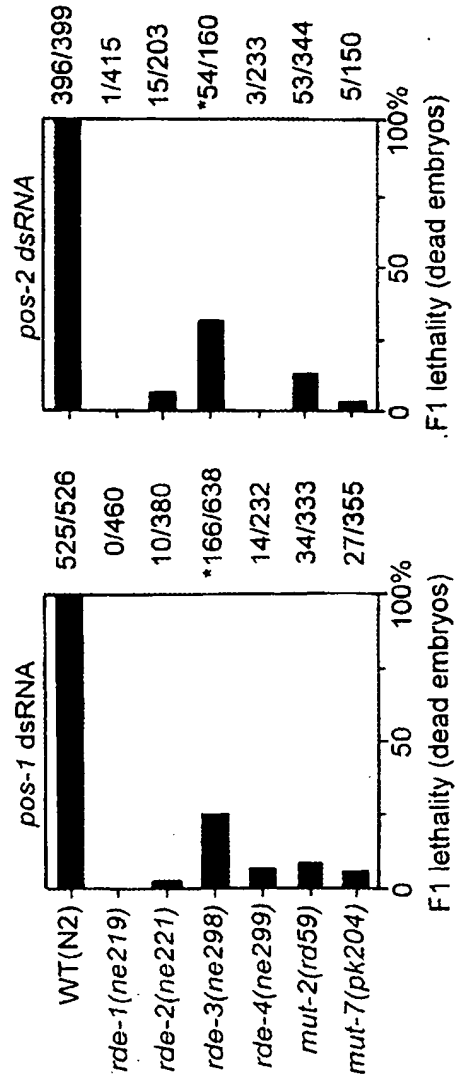
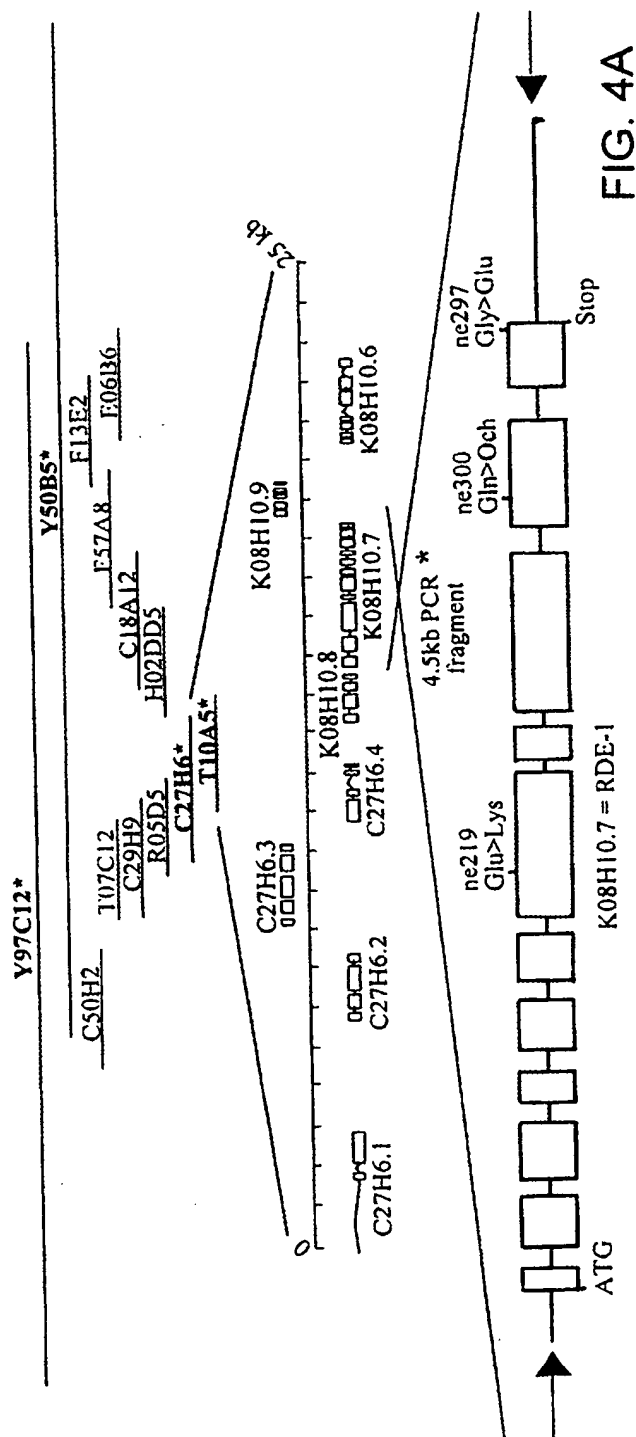


FIG. 2B

4/21



5/21

```

RDE-1      203 VN-----EIKVQANRNFVYDNNSSILNVPZSFHDENRPFQSDVAPRFAWFGIIGIKELDGEVLN-----
F48F7.1    235 S-----AVEQFSLFVGVQSTEDNVEEYQCPHFKVQNDVNR--EHSVYEVHSSEHVPNASSVMAGSCPPQASGAV
          77  -----GKDRIFATINSCVSLQAHDLISGLPSVPSPTICADVAPR--EHEPFAVHSPTASLEG-----C2
ZWILLE     226 GIINGPKREESYVANEVARANNHNGEFLAKRADCEPQAYOHITVIR--EHSVDFCRVGSSESYDIK-----
Sting      189 -----RAGENIEIKKAVESYQNTDAQFQVNLPRAMEGDLKLSEYIIDQANIN-----

RDE-1      267 -----FAIVDKFTYNAPRMSLLDYLLIVDPQSENDDVQDQTKLMAGKQTIROAAPRIQ-----GLENILKLCAL
F48F7.1    319 AGGAHSAGQYHAESKIGGGEZVWTFQHSYRPSQNSINDESRATRSRSEVETIATVEELFVQALAEPRASDAQVETVIRKGL
          144 -----NR-----GGORVETVQVQVRELELNMV--SAG--EACV--EFCDFKSIIEQQQ--PIDEQV--EFGK
          297 -----TEQRIGESLSHCQYQSTERTQCGSLNIDMASAFIEPDEVEEVAQLLQGVLSKPLS-----PSDAVIRKQIRGV
          244 -----LENERMQLPQYOTSQCHENDILLCSIECHVYVGTETILYNILSDAIRSDDDYQSTFK-----R-VY

          K ne219
RDE-1      336 VVDNEMSRLLTERHILTFLECEENSLVYVTGKSD---RGRNANKYDITLFKINENKATFEFPHLELVKSGATYAYVMEHLEVHCKPO
F48F7.1    409 KIEITHCQREKSRVENVTRPOTOTHTIGL---ETQHIETVQVQVNDKRIQKPHLPGLQVGOQKMTTPPEVQCNIVP-GO
          220 KIEITHCQREKSRVENVTRPOTOTHTIGL---ETQHIETVQVQVNDKRIQKPHLPGLQVGOQKMTTPPEVQCNIVP-GO
          372 KVEVITRANVREYVAGUTQRELELTVVD-----EN---CTKMSVLEEQSGFTQHTLPCLVGVQKASIEPMEKRIVE-GO
          305 MGVVILTDDYNNKTYAIDVDFQSPLCETKNDGZISYVDYVGRYNIIRDLPQVMSRFDKNIRGQNDQAIMILPELRATGN-TD

RDE-1      424 RYKNRIDLVMQDKFKEKATSKPHYKNTETMLKELDESSBELNEVEREGLCSLQSTIECPKVKKEP--VNSVDEQIK--TPVIRGCEK
F48F7.1    494 QETKELVDQETPMKATASAPTEKREISENLVRAVESAD---PQTHESQENPTEHETGRVISADNDLQSTR--ATLNGCVTDH
          305 RQITQVNDQNTBMRNESAEDQERSEKMSSENTD---VVRREGVDEEDIDVLOPSEKISGSKNK--NLTAVQVTDH
          454 RYTKQENKGI TALKVTCORREGQNDLEATVQNAIDQD---EHLAEGTSEKISSEJAEIETAEKTHGEGEDCLQVQVQNN
          394 AMRADFTLRANSEHTLNPDRRIETLRVFNKRLKSCQSV---ETLKSWNFEIDSNAVEIPANVPEEKEFQKNOKIFVCDARADWTNEF

```

FIG. 4B-1

6/21

RDE-1 514 QLNWPEKELCCAVENNETAGNPCLENDVVKETTEIGGKFRGIEPCANENRGAQSIMYDANKNEAFYKNCITLNTGIGRFEIAATE
F48F7.1 581 RQOQHIGNDPRWATACLEAQ-QQHYKENDIN-FAQCQRENDNGGATVGNPCERKAVGVGEQVZFMTRYKONYSQ-----
eIF2C 392 RNKQFHIGERKVAACQAP-PROCTEVHUKSETEDRKTEHOGGATQOPKQKQAPASVGPEDKANTALG-----
ZWILLE 542 MNRQINGMTISRACVNF-----SSQENVAGCECEELGECVSGERFNPVIEISARPDQVEKALSHVYHTSNKTK-----
Sting 482 RTCSFKGVHINRYVITPS-----RNLR-EAQSEVEGRTASSKMAICNPEDEIDDRNGTSSAIDNAN-----
RDE-1 604 AKNMFERLPDKEQKVMFIIISKRQLNANGVVKHYGCHTIGVANCHITSETVVALASLRHEKSGKRIFVQLAKKINAKLGGINOEJWS
F48F7.1 658 -----GQMAATGEG-KETVWVRKVGATZGTAOCQAKNAIRITY-----CTENKGLKKNKLCQONSIFLHN
eIF2C 469 -----QMAATGEG-KETVWVRKVGATZGTAOCQAKNAIRITY-----CTENKGLKKNKLCQONSIFLHN
ZWILLE 620 -----GKLELLAEDDNGSILKDKHTEKEIGLITQOGLTHMTNISK-----HYLADVLEKIN-KXGGRNTVEVDA
Sting 552 -----DQGIYVYVRSPEEKYSICIKRTCDVRPPEOVVETLVIAPRQQ-----KETGLMSIATKVIQNNAKLNGAP
RDE-1 694 EIAEISPEEKERTMPLTGVGIDVTHPTSYSCIDYSIAAVASINPGGT-IRNMIVTQEECRPGERAVAHGRE---RTDILEAKFVK
F48F7.1 725 V-----RPIETNEPAGGCDIHHPRAGDSRKSIAAAVSSQKHPES-FAATVYCOHROETSDITY-----KATF
eIF2C 536 G-----RPIVTOQVAGGCDVTHPTSYSCISIAAAVSSQKHPES-FAATVYCOHROETSDITY-----KATF
ZWILLE 691 IS-----CRIELSDIETIIIGDVTHPENGEESSESIAAAVSSQKHPES-FAATVYCOHROETSDITY-----KATF
Sting 621 W-----QVVPLHGLTVGSDVCHSP--KNENKSYCAEVAWQOKES-FFSTNEHINKQESSEQMSVN-----MAC

FIG. 4B-2

7/21

* ne300

RDE-1	780	LRSEAEINNDNRADAHIVVYRDGVSDSEMLRVSDELRSRSEVVKQFMSERDGEDPEPKYTEIVOKRHHNTRILRRMEKOKPVPVWKDLTP
F48F7.1	792	LLVGLKRNTRINPAPILVYRDGVSEKAFNMQVLAHETACATRCYOGG--TFEIVOKRHHNTRILRAVERKDO--V--
gIF2C	603	LLTQKTKSRRKPTTIRYRDGVSECGQVDFEELARAKIKEDQFG--TFEIVOKRHHNTRILCTEYER--V--
ZWILLE	775	LLISRRKAG-CRPLIRYRDGVSECGQVDFEELARAKIKEDQFG--TFEIVOKRHHNTRILANNHRDNRST--
Sting	687	ALERTQOHR-SLPEIRIFRDGVGDQOLAVNSEVNTKDRLEIYAGKQEGC-RKFEIIVSKRINSSETGHRNPV--
RDE-1	870	AETDVAAAVKQWEEDMKESKETGIVNPSSGTTVDKLLVSKYKEDFFLASRPGVLTGSRPCHYVVMXDDKGSGDEVYKQTYGLAFLSAR
F48F7.1	868	-----QVAYNREPSTGTTVDVGHITETFDLSSHGCGCTSRPEHYVMXDDNINLADKCTTYGCHTAR
gIF2C	679	-----QVAYNREPSTGTTVDVGHITETFDLSSHGCGCTSRPEHYVMXDDNINLADKCTTYGCHTAR
ZWILLE	853	-----DRSENHLPTSTVVDVGHITETFDLSSHGCGCTSRPEHYVMXDDNINLADKCTTYGCHTAR
Sting	766	-----E-CTVDDVVIPIERYDEELVSGVRICTVSTSNVISNMGENADKIDHSVWTHMXIN
RDE-1	960	CRKPISEVPMVYAHLSCEKAMELYRTYKHYIDYAOPTREHETELFQTNVKYPCGSEA--(SEQ ID NO: 13)
F48F7.1	936	CTRSVSEPEAPVYAHLVAFNARVHLVDREHDCGTSOPHCESEDITLSNAPAYONILANLVSI--(SEQ ID NO: 9)
gIF2C	747	CTRSVSEPEAPVYAHLVAFNARVHLVDREHDCGTSOPHCESEDITLSNAPAYONILANLVSI--(SEQ ID NO: 10)
ZWILLE	921	CTRSVSEPEAPVYAHLVAFNARVHLVDREHDCGTSOPHCESEDITLSNAPAYONILANLVSI--(SEQ ID NO: 6)
Sting	827	YSGTIRVEVYCVHANKLLVLAESINRAPSAGLQQLYFL--(SEQ ID NO: 7)

FIG. 4B-3

8/21

caggccacaaagtgatgaaacatgctccgaaatttcccgaaattggaaaaaggattttatcgtcattctctcgatccgggtat
 tgaatcattattagcagctataagatatataagtttgaataataataatataaggaatgaataatggccttgcgaggccccactg
 cttaaatggcagcggcnaaatctatgagagaaagtaactctcttgggtaaatggttcaagttctccagcnaaaatttacgat
 cggggataatacagagtatgaaagtgaanaaagacaaaggaaagtattgaaatagaaacccaggaaaacccttcccaaaaaag
 agaaaattcccaatgtaaaggcttgtaaaattagtaaaaactaattttatttttcagtcgccgatacgtgcnaaaactctctggc
 aaacatcttcggcagagagagagcagcagatatttctcgaagactatgtttttgtagtaaaaggacactgttttatagt
 cttttgcgaactgaaacctgtcaatcaaaaatgtgcctgggttcggagaaagttagtaaaaaggatttcggagaaaaaagatg
 aaaaagtattggagaaaaaaaatcttatacaatgatacttacctatcgtaaaaaaatctcacctgaaacttttagtcgagagaaa
 atccggaaaaaagacgaaagaaagcgaatcggaggttacaaaattctcgaaggtttatgaaaaaacacgcattataacaaacaa
 tttagcttcaggaatggtatgaccccaagaaagttcgtacgccttttggaaacgagagagattaaagtgtaggttgcaata
 aataataataataacaccctcaactcattatataattttaagaacaattcgcaaaaatttggtagcgaataataattcaat

FIG. 5A

9/21

tcctgcgagttccctgaatcgtttcacgatccaaacagattcgaacaatcattagaagtagcaccaagaatcgaagcatggt
ttggaatttacattggaatcaagaattgttcgatgggaacctgtgctcaattttgcaagtaagttgagaaactgcga
taaaaaatcatgtgattttgtgaagtgtcgataaaactattctacaatgcaccgaaaatgtctcttcgtgattatctt
ctcctaattgtcgacccccagtcgtgtaacgatgatgtacgaaaagatcttaaaacaaaactgatggcgggaaaaatgac
aatcagacaagccgcgcccgaagaattcgacaattattggaaaaattgaagctgaaatgcgcagaagttgggataac
aaatgttagtttaaatatttcaacaartaataracaaattgattttcaggtcgagattgacagaacgacatctgacatt
tctagattttgtcgaggaaaaactctctgtttataaagtcactggtaaatcggacagaggaagaaatgcaaaaaagracg
atactacattgttcaaaatctatgaggaaaaacaaaagttcattgagtttccccacctaccactagtcaaaagttaaagt
ggagcaaaagaatacgtgtaccaatggaacatcttgaagttcatgagaagccacaaagatacaagaatcgaattgatc
gggtgatgcaagacaagtttctaagcgcgacacgaaaaccctcgcactacaagaaaataccctaaaaatgctgaaa
aattggattttctcttctgaagagctaaattttgtgaaagatttgattatgctccaaacttcagatgatcgaatgtcca
ggaaagggtttgaaagagccaatgcttgtgaatagtgtaaatgaacaaattaaatgacaccagtgattcgtggatttca
agaaaaacaattgaatgtggttcccgaaaaagaactttgtgtgctgtttttgtagtcaacgaaacagcgggaaatccat
gcttagaagagaacgacggtgtgtaagtgtttctacgtagattattccgaaataatttcagtaagttctacaccgaact
aatgtgtgttgcaagttccgtggaatcgaattggtgccaatgaaaaacagaggagcgcaatctattatgtacgacgcga
cgaaaaatgaatatgccgtaagtttcagaaaaattgaaagtttttaatatcatatttacagttctacaaaaattgtacac
taaaraccggaatcggttagatttgaatatgccgcaacagaagcgaagaatgatgttgaacgtttcccgataaagaaca
aaagctttaatgttcattatcatttccaaacgacaactgaatgcttacgggtttgtgaaacattattgcgacacaccat
cgggtgtagctaatcagcatattacttctgaaacagtcacaaaagctttggcctcactaaggcagcagaaggatcaaaac
gaattttctatcaaatgtcattgaaaaatcaacgcgaaattaggaggtattaaccaggagcttgactggtcagaaattgca
gaaatatcaccagaagaaaaagaaagacggaaaaacaatgccattaactatgtatgttggaattgatgtaactcatccaa
ctctacagtggaattgattattctatagcggctgtagtagcgagtatcaatccaggtggaactatctatcgaaatatga
ttgtgactcaagaagaatgtcgtcccggtgagcgtgcagtggtctatggacgggaaagaacagatattttggaagcaaa
ttcgtgaaattgctcagagaattcgcagaagtgtgtgttcttgagtatttaaaagatctctgggattttaattttttg

FIG. 5B

SUBSTITUTE SHEET (RULE 26)

1
 2
 3
 4
 5
 6
 7
 8
 9
 10
 11
 12
 13
 14
 15
 16
 17
 18
 19
 20
 21
 22
 23
 24
 25
 26
 27
 28
 29
 30
 31
 32
 33
 34
 35
 36
 37
 38
 39
 40
 41
 42
 43
 44
 45
 46
 47
 48
 49
 50
 51
 52
 53
 54
 55
 56
 57
 58
 59
 60
 61
 62
 63
 64
 65
 66
 67
 68
 69
 70
 71
 72
 73
 74
 75
 76
 77
 78
 79
 80
 81
 82
 83
 84
 85
 86
 87
 88
 89
 90
 91
 92
 93
 94
 95
 96
 97
 98
 99
 100
 101
 102
 103
 104
 105
 106
 107
 108
 109
 110
 111
 112
 113
 114
 115
 116
 117
 118
 119
 120
 121
 122
 123
 124
 125
 126
 127
 128
 129
 130
 131
 132
 133
 134
 135
 136
 137
 138
 139
 140
 141
 142
 143
 144
 145
 146
 147
 148
 149
 150
 151
 152
 153
 154
 155
 156
 157
 158
 159
 160
 161
 162
 163
 164
 165
 166
 167
 168
 169
 170
 171
 172
 173
 174
 175
 176
 177
 178
 179
 180
 181
 182
 183
 184
 185
 186
 187
 188
 189
 190
 191
 192
 193
 194
 195
 196
 197
 198
 199
 200
 201
 202
 203
 204
 205
 206
 207
 208
 209
 210
 211
 212
 213
 214
 215
 216
 217
 218
 219
 220
 221
 222
 223
 224
 225
 226
 227
 228
 229
 230
 231
 232
 233
 234
 235
 236
 237
 238
 239
 240
 241
 242
 243
 244
 245
 246
 247
 248
 249
 250
 251
 252
 253
 254
 255
 256
 257
 258
 259
 260
 261
 262
 263
 264
 265
 266
 267
 268
 269
 270
 271
 272
 273
 274
 275
 276
 277
 278
 279
 280
 281
 282
 283
 284
 285
 286
 287
 288
 289
 290
 291
 292
 293
 294
 295
 296
 297
 298
 299
 300
 301
 302
 303
 304
 305
 306
 307
 308
 309
 310
 311
 312
 313
 314
 315
 316
 317
 318
 319
 320
 321
 322
 323
 324
 325
 326
 327
 328
 329
 330
 331
 332
 333
 334
 335
 336
 337
 338
 339
 340
 341
 342
 343
 344
 345
 346
 347
 348
 349
 350
 351
 352
 353
 354
 355
 356
 357
 358
 359
 360
 361
 362
 363
 364
 365
 366
 367
 368
 369
 370
 371
 372
 373
 374
 375
 376
 377
 378
 379
 380
 381
 382
 383
 384
 385
 386
 387
 388
 389
 390
 391
 392
 393
 394
 395
 396
 397
 398
 399
 400
 401
 402
 403
 404
 405
 406
 407
 408
 409
 410
 411
 412
 413
 414
 415
 416
 417
 418
 419
 420
 421
 422
 423
 424
 425
 426
 427
 428
 429
 430
 431
 432
 433
 434
 435
 436
 437
 438
 439
 440
 441
 442
 443
 444
 445
 446
 447
 448
 449
 450
 451
 452
 453
 454
 455
 456
 457
 458
 459
 460
 461
 462
 463
 464
 465
 466
 467
 468
 469
 470
 471
 472
 473
 474
 475
 476
 477
 478
 479
 480
 481
 482
 483
 484
 485
 486
 487
 488
 489
 490
 491
 492
 493
 494
 495
 496
 497
 498
 499
 500
 501
 502
 503
 504
 505
 506
 507
 508
 509
 510
 511
 512
 513
 514
 515
 516
 517
 518
 519
 520
 521
 522
 523
 524
 525

(SEQ ID NO:1)

cattccatgactaacgttttcataaattacttgaattt (SEQ ID NO:1)

11/21

CAGCCACAAAGTGATGAAC- 3' UTR

1/1	31/31
ATG TCG TCG AAT TTT CCG GAA TTG GAA AAA GGA TTT TAT CGT CAT TCT CTC GAT CCG GAG	
Met ser ser asn phe pro glu leu glu lys gly phe tyr arg his ser leu asp pro glu	
61/21	91/31
ATG AAA TGG CTT GCG AGG CCC ACT GGT AAA TGC GAC GGC AAA TTC TAT GAG AAG AAA GTA	
met lys trp leu ala arg pro thr gly lys cys asp gly lys phe tyr glu lys lys val	
121/41	151/51
CTT CTT TTG GTA AAT TGG TTC AAG TTC TCC AGC AAA ATT TAC GAT CCG GAA TAC TAC GAG	
leu leu leu val asn trp phe lys phe ser ser lys ile tyr asp arg glu tyr tyr glu	
181/61	211/71
TAT GAA GTG AAA ATG ACA AAG GAA GTA TTG AAT AGA AAA CCA GGA AAA CCT TTC CCA AAA	
tyr glu val lys met thr lys glu val leu asn arg lys pro gly lys pro phe pro lys	
241/81	271/91
AAG ACA GAA ATT CCA ATT CCC GAT CGT GCA AAA CTC TTC TGG CAA CAT CTT CCG CAT GAG	
lys thr glu ile pro ile pro asp arg ala lys leu phe trp gln his leu arg his glu	
301/101	331/111
AAG AAG CAG ACA GAT TTT ATT CTC GAA GAC TAT GTT TTT GAT GAA AAG GAC ACT GTT TAT	
lys lys gln thr asp phe ile leu glu asp tyr val phe asp glu lys asp thr val tyr	
361/121	391/131
AGT GTT TGT CGA CTG AAC ACT GTC ACA TCA AAA ATG CTG GTT TCG GAG AAA GTA GTA AAA	
ser val cys arg leu asn thr val thr ser lys met leu val ser glu lys val val lys	
421/141	451/151
AAG GAT TCG GAG AAA AAA GAT GAA AAG GAT TTG GAG AAA AAA ATC TTA TAC ACA ATG ATA	
lys asp ser glu lys lys asp glu lys asp leu glu lys lys ile leu tyr thr met ile	
481/161	511/171
CTT ACC TAT CGT AAA AAA TTT CAC CTG AAC TTT AGT CGA GAA AAT CCG GAA AAA GAC GAA	
leu thr tyr arg lys lys phe his leu asn phe ser arg glu asn pro glu lys asp glu	
541/191	571/191
GAA GCG AAT CCG AGT TAC AAA TTC CTG AAG AAT GTT ATG ACC CAG AAA GTT CCG TAC GCG	
glu ala asn arg ser tyr lys phe leu lys asn val met thr gln lys val arg tyr ala	
601/201	631/211
CCT CTT GTG AAC GAG GAG ATT AAA GTA CAA TTC GCG AAA AAT TTT GTG TAC GAT AAT AAT	
pro phe val asn glu glu ile lys val gln phe ala lys asn phe val tyr asp asn asn	
661/221	691/231
TCA ATT CTG CGA GGT CCG GAA TCG TTT CAC GAT CCA AAC AGA TTC GAA CAA TCA TTA GAA	
ser ile leu arg val pro glu ser phe his asp pro asn arg phe glu gln ser leu glu	
721/241	751/251

FIG. 6A

SUBSTITUTE SHEET (RULE 50)

12/21

ATA GCA CCA AGA ATC GAA GCA TGG TTT GGA ATT TAC ATT GGA ATC AAA GAA TTS TTC GAT
val ala pro arg ile glu ala trp phe gly ile tyr ile gly ile lys glu leu phe asp

731/261 811/271
GGT GAA CCT GTG CTC AAT TTT GCA ATT GTC GAT AAA CTA TTC TAC AAT GCA CCG AAA ATG
gly glu pro val leu asn phe ala ile val asp lys leu phe tyr asn ala pro lys met

841/291 971/291
TCT CTT CTG GAT TAT CTT CTC CTA ATT GTC GAC CCC CAG TCG TGT AAC GAT GAT GTA CGA
ser leu leu asp tyr leu leu leu ile val asp pro gln ser cys asn asp asp val arg

901/301 931/311
AAA GAT CTT AAA ACA AAA CTG ATG GCG GGA AAA ATG ACA ATC AGA CAA GCC GCG CGG CCA
lys asp leu lys thr lys leu met ala gly lys met thr ile arg gln ala ala arg pro

961/321 991/331
AGA ATT CGA CAA TTA TGG GAA AAT TTG AAG CTG AAA TGC GCA GAA GTT TGG GAT AAC GAA
arg ile arg gln leu leu glu asn leu lys leu lys cys ala glu val trp asp asn glu

1021/341 1051/351
ATG TCG AGA TTG ACA GAA CGA CAT CTG ACA TTT CTA GAT TTG TGC GAG GAA AAC TCT CTT
met ser arg leu thr glu arg his leu thr phe leu asp leu cys glu glu asn ser leu

1081/361 1111/371
GTT TAT AAA GTC ACT GGT AAA TCG GAC AGA GGA AGA AAT GCA AAA AAG TAC GAT ACT ACA
val tyr lys val thr gly lys ser asp arg gly arg asn ala lys lys tyr asp thr thr

1141/381 1171/391
TTG TTC AAA ATC TAT GAG GAA AAC AAA AAG TTT ATT GAG TTT CCC CAC CTA CCA CTA GTC
leu phe lys ile tyr glu glu asn lys lys phe ile glu phe pro his leu pro leu val

1201/401 1231/411
AAA GTT AAA AGT GGA GCA AAA GAA TAC GCT GTA CCA ATG GAA CAT CTT GAA GTT CAT GAG
lys val lys ser gly ala lys glu tyr ala val pro met glu his leu glu val his glu

1261/421 1291/431
AAG CCA CAA AGA TAC AAG AAT CGA ATT GAT CTG GTG ATG CAA GAC AAG TTT CTA AAG CGA
lys pro gln arg tyr lys asn arg ile asp leu val met gln asp lys phe leu lys arg

1321/441 1351/451
GCT ACA CGA AAA CCT CAC GAC TAC AAA GAA AAT ACC CTA AAA ATG CTG AAA GAA TTG GAT
ala thr arg lys pro his asp tyr lys glu asn thr leu lys met leu lys glu leu asp

1381/461 1411/471
TTC TCT TCT GAA GAG CTA AAT TTT GTT GAA AGA TTT GGA TTA TGC TCC AAA CTT CAG ATG
phe ser ser glu glu leu asn phe val glu arg phe gly leu cys ser lys leu gln met

1441/481 1471/491
ATC GAA TGT CCA GGA AAG GTT TTG AAA GAG CCA ATG CTT GTG AAT AGT GTA AAT GAA CAA
ile glu cys pro gly lys val leu lys glu pro met leu val asn ser val asn glu gln

1501/501 1531/511
ATT AAA ATG ACA CCA GTG ATT CGT GGA TTT CAA GAA AAA CAA TTG AAT GTG GTT CCC GAA
ile lys met thr pro val ile arg gly phe gln glu lys gln leu asn val val pro glu

FIG. 6B

13/21

1561/521 1591/531
AAA GAA TTT TCC TGT GGT GTT TTT GTA GTC AAC GAA ACA GCG GGA AAT GCA TGC TTA GAA
lys glu leu cys cys ala val phe val val asn glu thr ala gly asn pro cys leu glu

1621/541 1651/551
GAG AAC GAC GTT GTT AAG TTC TAC ACC GAA CTA ATT GGT GGT TCC AAG TTC CGT GGA ATA
glu asn asp val val lys phe tyr thr glu leu ile gly gly cys lys phe arg gly ile

1681/561 1711/571
CGA ATT GGT GCC AAT GAA AAC AGA GGA GCG CAA TCT ATT ATG TAC GAC GCG ACG AAA AAT
arg ile gly ala asn glu asn arg gly ala gin ser ile met tyr asp ala thr lys asn

1741/581 1771/591
GAA TAT GCC TTC TAC AAA AAT TGT ACA TTA AAT ACC GGA ATC GGT AGA TTT GAA ATA GCC
glu tyr ala phe tyr lys asn cys thr leu asn thr gly ile gly arg phe glu ile ala

1801/601 1831/611
GCA ACA GAA GCG AAG AAT AGC TTT GAA CGT CTT CCC GAT AAA GAA CAA AAA GTC TTA ATG
ala thr glu ala lys asn met phe glu arg leu pro asp lys glu gin lys val leu met

1861/621 1891/631
TTC ATT ATC ATT TCC AAA GCA CCA CTG AAT GCT TAC GGT TTT GTG AAA CAT TAT TGC GAT
phe ile ile ile ser lys arg gin leu asn ala tyr gly phe val lys his tyr cys asp

1921/641 1951/651
CAC ACC ATC GGT GTA GCT AAT CAG CAT ATT ACT TCT GAA ACA GTC ACA AAA GCT TTG GCA
his thr ile gly val ala asn gin his ile thr ser glu thr val thr lys ala leu ala

1981/661 2011/671
TCA CTA AGG CAC GAG AAA GGA TCA AAA CGA ATT TTC TAT CAA ATT GCA TTG AAA ATC AAC
ser leu arg his glu lys gly ser lys arg ile phe tyr gin ile ala leu lys ile asn

2041/681 2071/691
GCG AAA TTA GGA GGT ATT AAC CAG GAG CTT GAC TGG TCA GAA ATT GCA GAA ATA TCA CCA
ala lys leu gly gly ile asn gin glu leu asp asp ser glu ile ala glu ile ser pro

2101/701 2131/711
GAA GAA AAA GAA AGA GCG AAA ACA ATG CCA TTA ACT ATG TAT GTT GGA ATT GAT GTA ACT
glu glu lys glu arg arg lys thr met pro leu thr met tyr val gly ile asp val thr

2161/721 2191/731
CAT CCA ACC TCC TAC AGT GGA ATT GAT TAT TCT ATA GCG GCT GTA GTA GCG AGT ATC AAT
his pro thr ser tyr ser gly ile asp tyr ser ile ala ala val val ala ser ile asn

2221/741 2251/751
CCA GGT GGA ACT ATC TAT CCA AAT ATG ATT GTG ACT CAA GAA GAA TGT CGT CCC GGT GAG
pro gly gly thr ile tyr arg asn met ile val thr gin glu glu cys arg pro gly glu

2281/761 2311/771
CGT GCA GTG GCT CAT GGA GCG GAA AGA ACA GAT ATT TTG GAA GCA AAG TTC GTG AAA TTG
arg ala val ala his gly arg glu arg thr asp ile leu glu ala lys phe val lys leu

2341/781 2371/791
CTC AGA GAA TTC GCA GAA AAC AAC GAC AAT CGA GCA CCA GCG CAT ATT GTA GTC TAT CGA
leu arg glu phe ala glu asn asn asp asn arg ala pro ala his ile val val tyr arg

FIG. 6C

SUBSTITUTE SHEET (RULE 6C)

14/21

2401/801 2431/811
 AAC GGA GTT AGC GAT TCG GAG ATG CTA CGT GTT AGT CAT GAT GAG CTT CGA TCT TTA AAA
 asp gly val ser asp ser glu met leu arg val ser his asp glu leu arg ser leu lys

2461/821 2491/831
 AGC GAA GTA AAA CAA TTC ATG TCG GAA CGG GAT GGA GAA GAT CCA GAG CCG AAG TAC ACG
 ser glu val lys gln phe met ser glu arg asp gly glu asp pro glu pro lys tyr thr
 2521/841 2551/851
 TTC ATT GTG ATT CAG AAA AGA CAC AAT ACA CGA TTG CTT CGA AGA ATG GAA AAA GAT AAG
 phe ile val ile gln lys arg his asn thr arg leu leu arg arg met glu lys asp lys

2581/861 2611/871
 CCA GTG GTC AAT AAA GAT CTT ACT CCT GCT GAA ACA GAT GTC GCT GTT GCT GCT GTT AAA
 pro val val asn lys asp leu thr pro ala glu thr asp val ala val ala ala val lys

2641/881 2671/891
 CAA TGG GAG GAG GAT ATG AAA GAA AGC AAA GAA ACT GGA ATT GTG AAC CCA TCA TCC GGA
 gln trp glu glu asp met lys glu ser lys glu thr gly ile val asn pro ser ser gly

2701/901 2731/911
 ACA ACT GTG GAT AAA CTT ATC GTT TCG AAA TAC AAA TTC GAT TTT TTC TTG GCA TCT CAT
 thr thr val asp lys leu ile val ser lys tyr lys phe asp phe phe leu ala ser his

2761/921 2791/931
 CAT GGT GTC CTT GGT ACA TCT CGT CCA GGA CAT TAC ACT GTT ATG TAT GAC GAT AAA GGA
 his gly val leu gly thr ser arg pro gly his tyr thr val met tyr asp asp lys gly

2821/941 2851/951
 ATG AGC CAA GAT GAA GTC TAT AAA ATG ACC TAC GGA CTT GCT TTT CTC TCT GCT AGA TGT
 met ser gln asp glu val tyr lys met thr tyr gly leu ala phe leu ser ala arg cys

2881/961 2911/971
 CGA AAA CCC ATC TCG TTG CCT GTT CCG GTT CAT TAT GCT CAT TTA TCA TGT GAA AAA GCG
 arg lys pro ile ser leu pro val pro val his tyr ala his leu ser cys glu lys ala

2941/981 2971/991
 AAA GAG CTT TAT CGA ACT TAC AAG GAA CAT TAC ATC GGT GAC TAT GGA CAG CCA CGG ACT
 lys glu leu tyr arg thr tyr lys gln his tyr ile gly asp tyr ala gln pro arg thr

3001/1001 3031/1011
 CGA CAC GAA ATG GAA CAT TTT CTC CAA ACT AAC GTG AAG TAC CCT GGA ATG TCG TTC GCA
 arg his glu met glu his phe leu gln thr asn val lys tyr pro gly met ser phe ala

3061/1021 3091/1031
 TAA CAT TTT GCA AAA GTG TCG CCC GTT TCA ATC AAA TTT TTC AAT TGT AGA TAT TGT ACT
 CCH (SEQ ID NO:3)

3121/1041 3151/1051
 TAC TTT TTT TTA AAG CCC GGT TTC AAA AAT TCA TTC CAT GAC TAA CGT TTT CAT AAA TTA

3181/1061
 CTT GAA ATT TTA AAA AAA AAA AAA AAA (SEQ ID NO:2)

FIG. 6D

SUBSTITUTE SHEET (RULE 60)

15/21

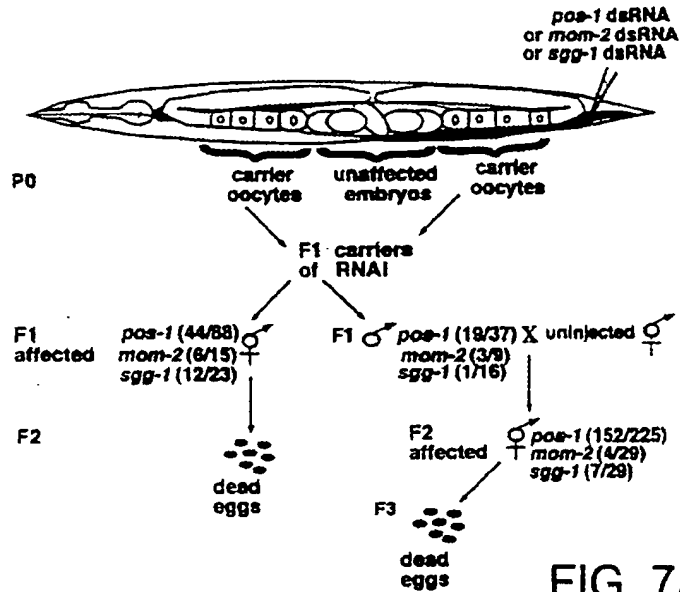


FIG. 7A

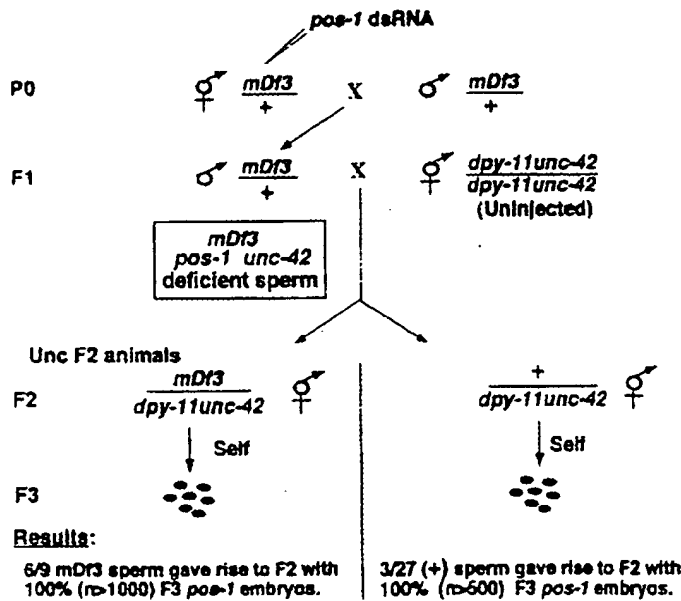


FIG. 7B

16/21





Injected P0	F1
 $\frac{\text{pos-1 dsRNA} \text{ } rde-1 \text{ unc-42}}{+}$	$\xrightarrow{\text{self X}}$ $rde-1 (-)$ 11/24 $rde-1 (+)$ 9/72
 $\frac{rde-2 \text{ unc-13}}{+}$	\longrightarrow $rde-2 (-)$ 0/39 $rde-2 (+)$ 23/78
 $\frac{mut-7 \text{ dpy-17}}{+}$	\longrightarrow $mut-7 (-)$ 0/15 $mut-7 (+)$ 20/50
 $\frac{rde-4 \text{ unc-69}}{+}$	\longrightarrow $rde-4 (-)$ 5/15 $rde-4 (+)$ 11/48

FIG. 8A



P0	Injected F1
 $\frac{rde-1 \text{ unc-42}}{+}$	$\xrightarrow{\text{pos-1 dsRNA}}$ $\frac{rde-1 \text{ unc-42}}{rde-1 \text{ unc-42}}$ 0/37
 $\frac{rde-4 \text{ unc-69}}{+}$	$\xrightarrow{\text{pos-1 dsRNA}}$ $\frac{rde-4 \text{ unc-69}}{rde-4 \text{ unc-69}}$ 0/37

FIG. 8B

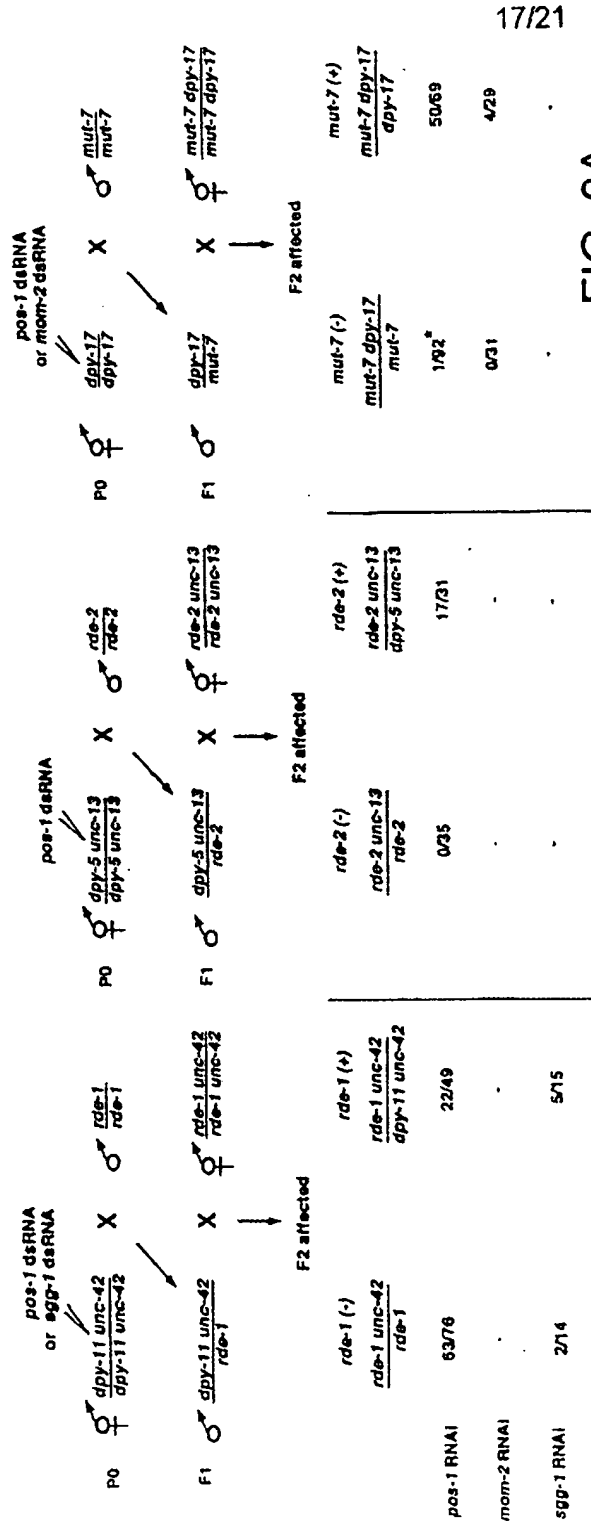


FIG. 9A

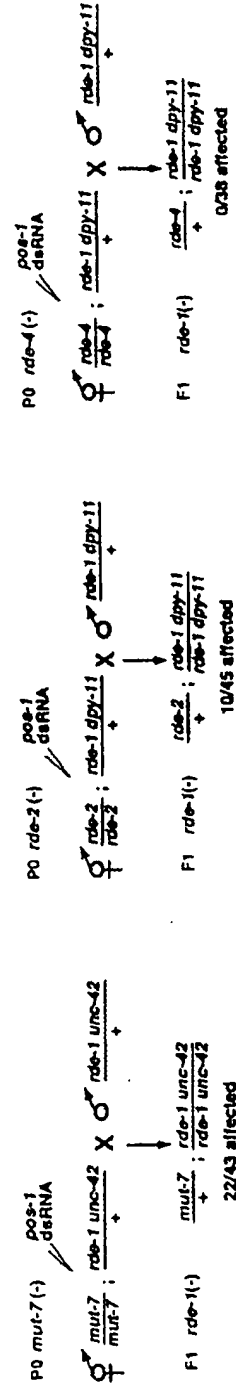


FIG. 9B

18/21

10 20 30 40 50 60
ATGGATTTAACCAAACTAACGTTTGAAAGCGTTTTCGGTGGATCAGATGTTCTTATGAAG
M D L T K L T F E S V F G G S D V P M K

70 80 90 100 110 120
CCTTCCCGATCGGAGGATAACAAAACGCCAAGAAACAGAACAGATTGGAGATGTTTCTG
P S R S E D N K T P R N R T D L E M F L

130 140 150 160 170 180
AAGAAAACCTCCCTCATGGTACTAGAAAGAGGCTGCTAAGGCTGCTATCAAAAGACGCCA
K K T P L M V L E E A A K A V Y Q K T P

190 200 210 220 230 240
ACTTGGGGCAGCTGTCGAACCTTCTGAAGGCTTCGAGATGACGTTGATTCTGAATGAAATT
T W G T V E L P E G F E M T L I L N E I

250 260 270 280 290 300
ACTGTAAAGGCCAGGCAACAAGCAAGAAAGCTGCGAGACAAAAGGCTGCTGTTGAATAT
T V K G Q A T S K K A A R Q K A A V E Y

310 320 330 340 350 360
TTACGCAAGGTTGTGGAGAAAGGAAAGCAGAAATCTTTTTCATTCTCGAACAACCAAA
L R K V V E K G K H E I F F I P G T T K

370 380 390 400 410 420
GAAGAAGCTCTTTTGAATATTGATCAAATATCGGATAAGGCTGAGGAATTGAAACGATCA
E E A L S N I D Q I S D K A E E L K R S

430 440 450 460 470 480
ACTTCAGATGCTGTTTCAGGATAACGATAACGATGATTTCGATTCTACAAGTCTGAATTT
T S D A V Q D N D N D D S I P T S A E F

490 500 510 520 530 540
CCACCTGGTATTTCGCCAACCGAGAATTGGGTGGGAAAGTTGCAGGAAAAATCTCAAAAA
P P G I S P T E N W V G K L Q E K S Q K

550 560 570 580 590 600
AGCAAGCTGCAAGCCCCAATCTATGAAGATTCCAAGAATGAGAGAACCGAGCGTTTCTTG
S K L Q A P I Y E D S K N E R T E R F L

610 620 630 640 650 660
GTTATATGCACGATGTGCAATCAAAAAACAGAGGAATCAGAAGTAAGAAGAAGGACGCA
V I C T M C N Q K T R G I R S K K K D A

670 680 690 700 710 720
AAGAATCTTGCAGCATGGTTGATGTGGAAGCGTTGGAAGACGGTATCGAATCTCTGGAA
K N L A A W L M W K A L E D G I E S L E

730 740 750 760 770 780
TCATATGATATGGTTGATGTGATGAAAATTTGGAAGAAGCTGAACATTTACTCGAAATT
S Y D M V D V I E N L E E A E H L L E I

FIG. 10A

19/21

```

      790      800      810      820      830      840
CAGGATCAAGCATCCAAGATTAAAGACAAGCATTCGGCACTGATTGATATACTCTCGGAC
Q D Q A S K I K D K H S A L I D I L S D

      850      860      870      880      890      900
AAGAAAAGATTTTCAGACTACAGCATGGATTTCAACGTATTATCAGTGAGCACAATGGGA
K K R F S D Y S M D F N V L S V S T M G

      910      920      930      940      950      960
ATACATCAGGTGCTATTGGAAATCTCGTTCCGGCGTCTAGTTTCTCCAGACCCCGACGAT
I H Q V L L E I S F R R L V S P D P D D

      970      980      990      1000      1010      1020
TTGGAAATGGGAGCAGAACACACCCAGACTGAAGAAATTATGAAGGCTACTGCCGAGAAG
L E M G A E H T Q T E E I M K A T A E K

      1030      1040      1050      1060      1070      1080
GAAAAGCTACGGAAGAAGATATGCCAGATTCCGGGCCGCTAGTGTTCCTGGACATGGT
E K L R K K N M P D S G P L V F A G H G

      1090      1100      1110      1120      1130      1140
TCATCGGCGGAAGAGGCTAAACAGTGTGCTTGTAAATCGGCGATTATCCATTTCACACC
S S A E E A K Q C A C K S A I I H F N T

      1150      1160      1170      1180      1190      1200
TATGATTTACGGATTGAAAATATTATTGCGTATTCCTGAAAAATGAAGCGTCTGAATGA
Y D F T D * K Y Y C V F L K N E A S E *

      1210      1220      1230
TTATAAAAAAAAAAAAAAAAAA (SEQ ID NO:4)
L * K K K K K (SEQ ID NO:5)

```

FIG. 10B

XIRBPA PVCSLOELAVQKGRRL-PEYTVAQESGPPHKEFTITCRVETF-VETSCGTSKQ-VAKRVAAEKLLTKF (SEQ ID NO: 11)
HSPKR FMEELNTYR-QKQGVV-LKYQELPNSGPPHDERFTFQVIIDGREFPECEGRSKK-EAKNAAAKLAVEIL (SEQ ID NO: 12)
RDE-4 WVGKLOEKS-QNSKLQAFIYEDSKN---ERTERFLVICIMCNQ-KTRGI-RSKKKDAKNIAAWLMWKA

20/21
 QE Y H F SKQ K
 CONSENSUS P---L-E---Q---Y---GP-H---F---V---G---G-G-SKK--AK--AA--AL--L (SEQ ID NO: 8)

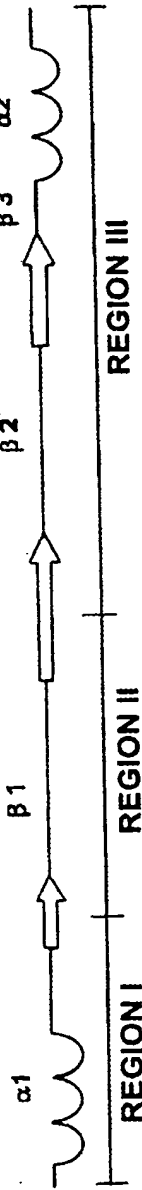
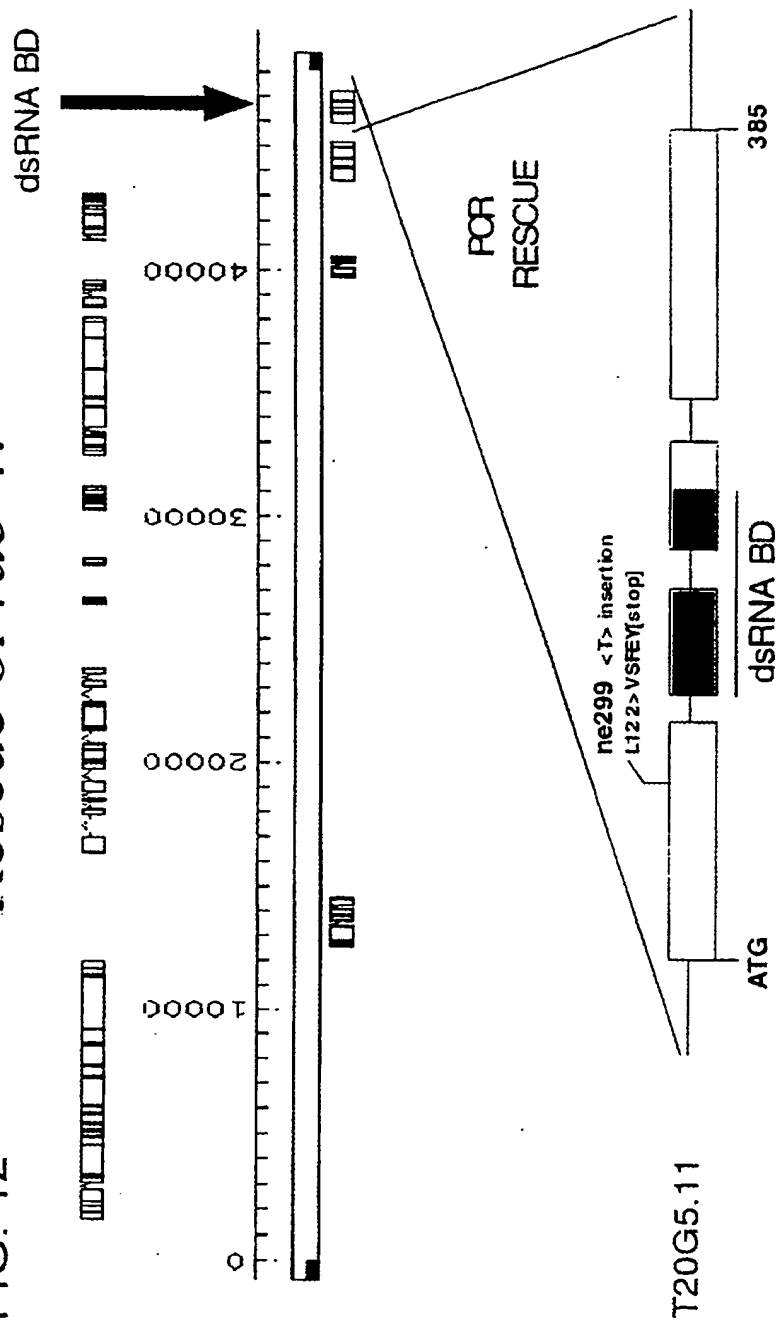


FIG. 11

FIG. 12 Rescue of *rde-4*:



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/28470

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/04; C07K 14/435, 16/18; C12N 15/63, 15/67

US CL : 536/23.1; 530/350, 387.1; 435/440

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 530/350, 387.1; 435/440

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN/CAS (Medline, Embase, Biosis, Caplus); US patent database

search terms: rna interference; dsRNA; RNAi; RNA interference mechanism; genetic interference; gene silencing; genetic basis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WATERSTON, R. et al. A survey of expressed genes in <i>Caenorhabditis elegans</i> . Nature Genetics. May 1992, Vol. 1, pages 114-123, especially pages 114-115.	1-3
X	WILSON, R. et al. 2.2 Mb of contiguous nucleotide sequence from chromosome III of <i>C. elegans</i> . Nature. 03 March 1994, Vol. 368, pages 32-38, especially pages 32-33.	8-10
X, P	TABARA, H. et al. The rde-1 gene, rna interference, and transposon silencing in <i>C. elegans</i> . Cell. 15 October 1999, Vol. 99, pages 123-132, especially pages 126-127.	1-3, 6, 7, 13

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be of particular relevance	* X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* A* document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means	
* P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

02 FEBRUARY 2001

Date of mailing of the international search report

19 MAR 2001

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

BRONWEN M. DOEB

Telephone No. (703) 308-1234

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/28470

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-4, 8-11 and 13, drawn to an isolated nucleic acid molecule and a method of using it.

Group II, claim(s) 5, drawn to an antibody that specifically binds RDE-1 polypeptide.

Group III, claim(s) 6 and 7, drawn to a method of enhancing the expression of a transgene in a cell.

Group IV, claim(s) 12, drawn to an antibody that specifically binds RDE-4 polypeptide.

Group V, claim(s) 14-16, drawn to a method of inhibiting the activity of a gene.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I is drawn to an isolated nucleic acid molecule which has the special technical feature of encoding an RDE-1 polypeptide. Group II is drawn to an antibody which has the special technical feature of binding specifically to RDE-1 polypeptide. Group III is drawn to a method of enhancing expression of a transgene in a cell which has the special technical feature of decreasing the activity of the RNAi pathway. Group IV is drawn to an antibody which has the special technical feature of binding specifically to RDE-4 polypeptide. Group V is drawn to a method of inhibiting the activity of a gene which has the special technical feature of introducing an RNAi agent into a cell.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.